Vascular Biology, Atherosclerosis and Endothelium Biology

A Key Role for the Integrin $\alpha 2\beta 1$ in Experimental and Developmental Angiogenesis

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The $\alpha 2\beta 1$ integrin receptor plays a key role in angiogenesis. Here we investigated the effects of small molecule inhibitors (SMIs) designed to disrupt integrin $\alpha 2$ I or $\beta 1$ I-like domain function on angiogenesis. In unchallenged endothelial cells, fibrillar collagen induced robust capillary morphogenesis. In contrast, tube formation was significantly reduced by SMI496, a $\beta 1$ I-like domain inhibitor and by function-blocking anti- $\alpha 2\beta 1$ but not - $\alpha 1\beta 1$ antibodies. Endothelial cells bound fluorescein-labeled collagen I fibrils, an interaction specifically inhibited by SMI496. Moreover, SMI496 caused cell retraction and cytoskeletal collapse of endothelial cells as well as delayed endothelial cell wound healing. SMI activities were examined *in vivo* by supplementing the growth medium of zebrafish embryos expressing green fluorescent protein under the control of the vascular endothelial growth factor receptor-2 promoter. SMI496, but not a control compound, interfered with angiogenesis *in vivo* by reversibly inhibiting sprouting from the axial vessels. We further characterized zebrafish $\alpha 2$ integrin and discovered that this integrin is highly conserved, especially the I domain. Notably, a similar vascular phenotype was induced by morpholino-mediated knockdown of the integrin $\alpha 2$ subunit. By live videomicroscopy, we confirmed that the vessels were largely nonfunctional in the absence of $\alpha 2\beta 1$ integrin. Collectively, our results provide strong biochemical and genetic evidence of a central role for

$\alpha 2\beta 1$ integrin in experimental and developmental angiogenesis. (Am J Pathol 2009, 175:1138–1347; DOI: 10.2353/ajpath.2009.090234)

Angiogenesis is the formation of new capillaries from pre-existing blood vessels and is essential for human development, wound healing, and tissue regeneration.¹ Angiogenesis is dependent on interactions of endothelial cells with growth factors and extracellular matrix components.^{2,3} Endothelial cell-collagen interactions are thought to play a role in angiogenesis *in vivo* and *in vitro* and require the function of the $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins,³ two receptors known to cross talk.⁴ Thus, vascular endothelial growth factor (VEGF)-induced angiogenesis in Matrigel plugs implanted in mice is markedly inhibited by anti- $\alpha 1\beta 1$ and - $\alpha 2\beta 1$ integrin antibodies.^{5,6} Studies using various collagen-induced angiogenesis assays also suggest a critical role for endothelial cell $\alpha 2\beta 1$ integrin^{2,7,8} binding to the GFPGER_{502–507} sequence of the collagen triple helix.⁹ Consistent with these findings, endorepellin, a potent anti-angiogenic molecule derived from the C terminus of perlecan^{10,11} disrupts $\alpha 2\beta 1$ integrin function,^{12–16} and some of the affected gene products have been associated with the integrin-mediated angiogenesis.¹⁷ Endothelial cell-collagen interactions may also contribute to tumor-associated angiogenesis.¹⁸ For example, gene products up-regulated in tumor-associated endothelial cells include types I, III, and VI collagens,¹⁹ and tumor-associated angiogenesis is sensitive to endorepellin treatment.^{15,20,21}

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Interestingly, $\alpha 2\beta 1$ integrin-null mice show no overt alteration in either vasculogenesis or angiogenesis but display only a mild platelet dysfunction phenotype and altered branching morphogenesis of the mammary glands.^{22,23} This observation suggests that in mammals, there is functional compensation during development, but that $\alpha 2\beta 1$ integrin might be required for postnatal angiogenesis. Indeed, when adult $\alpha 2\beta 1$ -null mice are experimentally challenged, they show an enhanced angiogenic response during wound healing²⁴ and tumor xenograft development.^{15,25}

The $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins include inserted domains (I domains) in their α subunits that mediate ligand binding.^{26,27} The $\alpha 2$ I domain is composed of a Rossman fold and a metal ion coordination site (MIDAS), proposed to ligate the GFPGER_{502–507} sequence of collagen, thereby inducing receptor activation.^{26,28} Other integrin domains may also play a role in ligand binding and receptor activation. For example, the $\beta 1$ I-like domain seems to allosterically modulate collagen ligation by the $\alpha 2$ I domain, and, intracellularly, the cytoplasmic sequence of the $\alpha 2$ subunit functions as a hinge, locking the receptor in an inactive conformation, and membrane-soluble peptide mimetics of this sequence were shown to promote $\alpha 2\beta 1$ receptor activation.²⁹ Recently, a family of small molecule inhibitors (SMIs)² targeting the function of the $\alpha 2\beta 1$ integrin were designed.³⁰ Specifically, inhibitors of $\alpha 2\beta 1$ integrin function were prepared using modular synthesis, enabling substitutions of arylamide scaffold backbones with various functional groups, creating SMIs targeted to the I domain or the intact integrin.^{30–32} In this study, we tested the activities of a group of SMIs on endothelial cell-collagen interactions and angiogenesis *in vitro* and *in vivo*. We provide evidence that SMI496, which binds between the I domains of $\beta 1$ and $\alpha 2$ subunits,³² interferes with $\alpha 2\beta 1$ integrin activity on endothelial cells both *in vitro* and *in vivo*, suggesting a potential therapeutic modality to interfere with angiogenesis. Moreover, interference with $\alpha 2$ integrin expression in embryonic zebrafish caused a vascular phenotype characterized by abnormal angiogenesis.

Materials and Methods

Small Molecule Inhibitors

SMIs were prepared using either solid-phase or solution-phase synthesis as described previously.³⁰ Final products were purified by preparative reverse-phase high-performance liquid chromatography using a GRACEVYDAC C-18 column and characterized by ¹H nuclear magnetic resonance spectroscopy, ¹³C nuclear magnetic resonance spectroscopy and electrospray ionization high-resolution mass spectroscopy.

Human Umbilical Vein Endothelial Cell Isolation and Culture

Umbilical cords were obtained from the Labor and Delivery Department, Thomas Jefferson University Hospital.

Endothelial cells were isolated from the cords as described previously.⁹ Endothelial cells were cultured on tissue culture flasks coated with 0.2% gelatin. Cells were fed complete media (Medium 199 [GIBCO, Grand Island, NY], 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 50 μ g/ml endothelial cell growth supplement, 50 μ g/ml heparin sodium salt from porcine intestinal mucosa (grade I-A, Sigma-Aldrich, St. Louis, MO), 1.0% penicillin-streptomycin, and 0.1% Fungizone (GIBCO). Endothelial cell growth supplement was isolated from bovine hypothalami.⁹ Endothelial cells were used through passage 6.

In Vitro Angiogenic Assays

For branching morphogenesis assays using a collagen sandwich, endothelial cells were plated at 10^5 cells/cm²/well onto 12-well plates coated with 100 μ g/ml type I collagen in 10 mmol/L acetic acid at 25 μ g/cm². Cells were then incubated overnight at 37°C and allowed to reach confluence. The next day the cells were rinsed and an apical collagen gel was applied to each well at 100 μ l/cm² (control wells received an equivalent volume of cold serum-free media). The collagen gel was made by mixing 70% 1.5 mg/ml type I collagen in 10 mmol/L acetic acid, 10% 10 $\times$ culture salts, and 20% 11.8 mg/ml sodium bicarbonate. After the gel was added, the plates were incubated for 15 minutes at 37°C to allow the gel to polymerize. After polymerization, warm serum-free media plus growth factors (VEGF and fibroblast growth factor-2, 5 ng/ml each; BD Biosciences, San Jose, CA),⁹ and any test agents were added to the wells. The cells were returned to the 37°C incubator, and micrographs were taken at 2, 4, 8, 12, and 24 hours after gel addition. For branching morphogenesis assays on Matrigel, endothelial cells were untreated or treated in suspension for 10 minutes with 50 to 100 nmol/L SMI496 or dimethyl sulfoxide. Endothelial cells were plated in complete media at 10^5 cells/well onto a four-well chamber slide precoated with Matrigel (BD Biosciences) and incubated at 37°C. Tube formation was assayed over the course of 3 to 4.5 hours.

Endothelial Cell Growth and Migration Assays

Endothelial cells were cultured on 96-well tissue culture plates at 10^4 cells/well overnight. Cell growth was then examined for 4 days in the presence of complete culture medium, culture medium lacking endothelial cell growth supplement, and heparin and complete culture medium plus SMI496, 488, 394, and 355, and an anti- $\alpha 2\beta 1$ function-blocking antibody (Chemicon International, Temecula, CA). Cell numbers were determined at various times by staining the cultures with crystal violet and extracting the dye with Triton X-100. The soluble dye was measured (A_{590}) using a SpectraMax 340PC microplate reader and SoftMax Pro 5 software to determine cell number based on endothelial cell standard curves. For cell migration, endothelial cells were plated at 10^5 cells/cm²/well onto 12-well plates coated with 100 μ g/ml type

I collagen in 10 mmol/L acetic acid at 25 $\mu\text{g}/\text{cm}^2$. On the third day, a "scrape wound" was made across the center of the well by dragging a 200- μl plastic pipette tip through the cell monolayer. The cells were rinsed with Dulbecco's phosphate buffered saline (Mediatech, Herndon, VA) and resupplemented with complete medium plus test agents. At various time points micrographs were taken of the wound area.

Flow Cytometry

This assay was adapted from a published protocol.²⁹ Endothelial cells, passages 4 to 6, were trypsinized and allowed to recover for 2 hours under serum-free conditions. After washing, the cells were treated with chemical inhibitors or inhibitory antibodies at concentrations of 50 and 100 $\mu\text{g}/\text{ml}$, respectively, in PBS+/1% bovine serum albumin. Without removing the inhibitors, collagen labeled with fluorescein 5-isothiocyanate (FITC) Sigma-Aldrich) was then added to cells at 50 $\mu\text{g}/\text{ml}$. A final incubation with 7-amino-actinomycin D (Sigma-Aldrich) allowed the detection of dead cells. Cells were then analyzed on a Beckman Coulter EPICS flow cytometer. The CD2 antibody and SMI723 were used to obtain baseline measures of FITC-collagen staining for the function blocking antibody and SMI496, respectively. The proportion of cells binding FITC-collagen at baseline was set to 1.0 for each group, and the effect of inhibitors was expressed as a ratio of the percentage of cells binding FITC-collagen in the presence of inhibitors divided by the percentage of cells binding FITC-collagen in the presence of a control inhibitor or antibody.

Endothelial Cell Attachment to Collagen Substrata

Endothelial cells were cultured on type I collagen films and exposed to conditioned medium or conditioned medium including SMI496 at 50 $\mu\text{g}/\text{ml}$ for 30 minutes. The cells were then fixed with 4.0% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with phalloidin-FITC to visualize actin stress fibers and counterstained with 4,6-diamidino-2-phenylindole to visualize nuclei.

Zebrafish Studies

We used wild-type or transgenic zebrafish expressing green fluorescent protein (GFP) under control of the VEGF receptor 2 (*vegfr2-gfp*)³³ or fli promoter (*fli1a:nEGFP*)³⁴. Embryos were housed within the zebrafish facility of Thomas Jefferson University and were raised at 28.5°C according to standard practice. Embryos were generated by natural pair-wise crossing, and all developmental staging was reported as days postfertilization (dpf). For *in vivo* zebrafish SMI angiogenesis assays, stock solutions of SMI496 and 355 (1.0 mg/ml) in water were directly added to zebrafish embryo medium to reach a final concentration of 4, 20, and 50 $\mu\text{mol}/\text{L}$, a range used previ-

ously to study small molecule anti-angiogenic compounds such as SU5416 and SU6668.³³ All experiments were repeated two times using at least 40 embryos per treatment. For the characterization of zebrafish $\alpha 2$ integrin, RT-PCR was performed on cDNA derived from pools of staged embryos ($n = 50$ embryos per group). Integrin $\alpha 2$ was detected using $\alpha 2$ forward primer 5'-CAGAGAATCGTTGGAGGGAACTG-3' and $\alpha 2$ reverse primer 5'-CGATTAGCCTGATAGAGTTTGTGG-3' (Invitrogen). PCR reactions were performed as described previously.³⁵ For the knockdown of zebrafish integrin $\alpha 2$, a morpholino (Gene Tools, LLC, Philomath, OR) was designed to target the 5'-untranslated region/translation start of zebrafish integrin $\alpha 2$ ($\alpha 2$ -MO). The morpholino sequence was 5'-CCAGTAGAATCACAGCCTTTTCCAT-3'. The standard control morpholino sequence was 5'-CCTCTTACCTCAGTTACAATTTATA-3'. The p53 co-knockdown experiment was performed to eliminate off-target morpholino effects as described previously.³⁶ Morpholinos (1–10 ng/embryo) were microinjected into one-cell wild-type or vascular transgenic embryos according to the standard protocol.³⁷ Subintestinal vessel development was assayed by endogenous alkaline phosphatase staining as described previously.³⁵ Embryos were studied on a Leica MZFIII stereomicroscope equipped with an Axiocam camera (Carl Zeiss Inc., Thornwood, NY) and AxioVision software version 3.0.6.1, a Leica MZ16FA stereomicroscope equipped with a DFC500 camera and Application Suite version 2.5.0.r1 (Leica, Wetzlar, Germany) or a Leica DM5500 B equipped with a DFC340 FX camera and LAS AF version 1.6.1 (Leica). Embryos were imaged in embryo media or 3 to 4% methyl cellulose and anesthetized with tricaine. Images were assembled using Adobe Photoshop CS2.

Statistical Analysis

All data were graphed and analyzed using the Sigma Plot and Sigma Stat software package. Statistical analysis between groups was assessed by Student's *t*-test. The data were considered statistically significant with $P < 0.05$.

Results

The goal of these experiments was to examine whether SMIs designed to disrupt $\alpha 2\beta 1$ integrin function would also influence integrin-collagen binding and collagen-induced or native angiogenesis *in vitro* and *in vivo* and to examine the role of $\alpha 2\beta 1$ integrin in developmental angiogenesis.

Selection of Small Molecule Inhibitors

We selected a variety of compounds as positive and negative controls for these studies. Compound SMI496 is a prototype inhibitor for $\alpha 2\beta 1$, with an IC_{50} for inhibition of platelet association to collagen I of approximately 10 nmol/L and was identified in a screen for compounds that

Figure 1. Structure of small molecule inhibitors of the $\alpha 2\beta 1$ integrin receptor. Low M_r (<600 Da) benzene scaffold derivatives designed to bind and functionally disrupt the $\alpha 2\beta 1$ integrin receptor. Inhibitors and their receptor target sites include the following: SMI355, 394, and 418, $\alpha 2$ domain; SMI488 and 723 (not shown) negative controls; and SMI496, $\beta 1$ -like domain.

inhibit $\alpha 2\beta 1$ -dependent adhesion of platelets to collagen³⁰ (Figure 1). SMI146 is highly selective for $\alpha 2\beta 1$ relative to $\alpha 1\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha v\beta 3$, and $\alpha IIb\beta 3$. Compound SMI488, a close analog of SMI496, has at least 100-fold lower affinity for $\alpha 2\beta 1$. The compounds SMI355, 394, 418, and 723 were very weakly active (affinities in the mid-mmol/L range), were ultimately found to act non-specifically, and were used as negative controls.

Effects of SMIs on Endothelial Cell Branching Morphogenesis

For these studies, angiogenesis was induced by an apical type I collagen gel placed on a confluent endothelial cell monolayer. In control cultures at 0 hours, cells are confluent, and after collagen gel addition, at 2 to 4 hours, cell movement and reorganization of the cell layer occurs; at 6 to 8 hours, cultures are about 50% reorganized; and at 12 to 24 hours, capillary tube formation is complete (Figure 2A). We have shown previously by electron microscopy that these tubes contain *bona fide* lumina.⁹ In contrast, cultures exposed to SMI496 or the anti- $\alpha 2\beta 1$ integrin antibody, but not to negative control SMIs, exhibited only a slight amount of cell movement and reorganization with very few gaps in the cell layer at all time points. Effects of the SMIs on angiogenesis at

Figure 3. Effects of SMIs on growth. Notice the lack of growth inhibition by all of the SMIs tested. By 4 days a borderline ($P = 0.061$) inhibition of cell growth by SMI496 was observed. Values represent the mean $\pm$ SE of three independent experiments run in triplicate. Endothelial cells were supplemented daily with fresh media containing the various SMIs (all at ~ 100 nmol/L) or $10 \mu\text{g/ml}$ of an anti- $\alpha 2\beta 1$ integrin function-blocking antibody. On each day, plates were fixed with glutaraldehyde and on day 4, all cultures were stained with crystal violet, the dye was detergent extracted, and cell number was determined spectrophotometrically.

various times up to 24 hours after collagen gel addition were quantified, confirming that only SMI496 and the anti- $\alpha 2\beta 1$ integrin antibody significantly ($P < 0.001$) inhibited tube formation in three independent experiments (Figure 2B). Examination of branching morphogenesis on Matrigel revealed that SMI496 did not block endothelial cell tube formation on this substrate (supplemental Figure S1, see <http://ajp.amipathol.org>).

Effects of SMIs on Endothelial Cell Growth

To ensure that the SMI496 effects were not due to cytotoxicity, we performed growth experiments in which endothelial cells were challenged with the various SMIs. The results showed no significant effect on cell growth by any of the SMIs for the first 2 days of culture (Figure 3), which exceeds the 1-day treatment period in which SMI496

Figure 2. Disruption of collagen-induced *in vitro* capillary morphogenesis by SMI496, an inhibitor of $\alpha 2\beta 1$ -integrin function. **A:** Photomicrographs of live endothelial cells undergoing *in vitro* capillary morphogenesis in serum-free defined media supplemented with VEGF-fibroblast growth factor-2 (5 ng/ml each) $\pm$ SMI496 (100 nmol/L). Note the complete block of capillary-like formation by SMI496, clearly visible at 24 hours. Similar results were obtained with an anti- $\alpha 2\beta 1$ integrin function-blocking antibody (5 μ g/ml). No effect was observed with any of the other SMIs (not shown). Scale bar = ~ 100 μ m. **B:** Quantification of three independent experiments similar to that shown in **A**. Each experiment was run in triplicate. Values represent the mean $\pm$ SE. Notice that only SMI496 is capable of blocking capillary formation (** $P < 0.001$). A similar inhibition was also evoked by an anti- $\alpha 2\beta 1$ integrin function-blocking antibody. Angiogenic areas were measured using the ImageJ program.

Figure 4. Effects of SMIs on endothelial cell-collagen fibril ligation in suspension. Note that SMI496 but not the negative compound SMI723 disrupts FITC-labeled collagen binding. The anti-α2β1 integrin function-blocking antibody but not an anti-CD2 blocking antibody has a similar effect. For flow cytometry studies, endothelial cells were trypsinized, allowed to recover for 2 hours, and then exposed to SMIs (50 nmol/L) or antibodies (100 μg/ml) in PBS ± 1% bovine serum albumin. FITC-collagen (50 μg/ml) was then added. Cell-FITC collagen interactions were analyzed on a Beckman Coulter EPICS flow cytometer. Anti-CD2 antibody and the negative control SMI723 were used to obtain baseline measures of FITC-collagen staining. Values represent the mean ± SE from three independent experiments run in triplicate (****P* < 0.001, by Student's *t*-test). Scatterplots are found in Supplemental Figure S2, see <http://ajp.amipathol.org>.

exerts its potent anti-angiogenic activity (Figure 2). Thereafter, there was some inhibition of growth evoked by SMI496 and growth promotion exerted by several other compounds (Figure 3). Thus, SMI496 does not seem to be cytotoxic or growth-active within the time frame that it is inhibitory to angiogenesis.

SMI496 Disrupts Endothelial Cell-Collagen Interactions

Next, we examined the effect of SMIs on endothelial cell interactions with type I collagen fibrils, either in suspension or immobilized on tissue culture plates. To study the interaction of collagen fibrils in suspension with endothelial cells, type I collagen fibrils were labeled with FITC such that they retained the capacity to promote angiogenesis *in vitro* (data not shown). Thus, under control conditions, approximately 80% of endothelial cells bound to FITC-collagen. Collagen binding was inhibited ~65% by SMI496 or the α2β1 function blocking antibody (*P* < 0.001) but not by SMI723 or an anti-CD2 antibody (Figure 4; supplemental Figure S2, see <http://ajp.amipathol.org>). When type I collagen was immobilized, endothelial cells displayed a well-spread cell morphology and a normal actin cytoskeleton with distinct and numerous cytoplasmic filamentous actin bundles (Figure 5A). However, treatment with SMI496 disrupted endothelial cell-collagen interactions: the cells were substantially less well spread and actin was now present in a punctate pattern indicative of a collapse of the actin cytoskeleton (Figure 5, B and C, arrows).

SMI496 Affects Endothelial Cell Migration

The effect of SMI496 on endothelial cell migration was tested using a scratch wound assay in which the ability of endothelial cells to migrate into the wounded area of a confluent culture was measured over time (Figure 5, D and E). At 24 hours, control cultures showed growth into the wound area (Figure 5F). However, endothelial cell migration was substantially delayed by adding SMI496 to the culture medium (Figure 5G). Quantification of two independent experiments performed in triplicate showed

Figure 5. Effects of SMIs on endothelial cell attachment to collagen substrata and migration. Fluorescent micrographs of endothelial cells cultured on type I collagen treated for 30 minutes with either vehicle (A) or SMI496 (100 nmol/L) (B and C). The arrows point to the collapse and coalescence of actin bundles. The asterisks point to areas of endothelial cell retraction. The cells were fixed, detergent-permeabilized, and stained with phalloidin-FITC (green) to visualize actin stress fibers and counterstained with DAPI (blue) to visualize nuclei. Note the partial collapse of the actin cytoskeleton (arrows, B and C). The experiments were repeated three times with similar results. Scale bar = 20 μm. D–G: Light micrographs of “wounded” endothelial monolayers and the effect of SMI496. Confluent endothelial cell layers on collagen films were scraped with a 200-μl pipette tip. The cells were rinsed with PBS and resupplemented with either media alone or media supplemented with SMI496 (100 nmol/L). Micrographs were taken at various time points. Scale bar = 100 μm. H: Quantification of endothelial cell wound closure over time. The areas within the scratches were quantified using ImageJ software and expressed as percentage of wound closure at time 0. Values represent the mean ± SE of two independent experiments run in triplicate.

Figure 6. SMI496 inhibits angiogenesis in the zebrafish trunk. **A–C:** Bright-field and fluorescent micrographs of live *vegfr2-gfp* zebrafish controls at 2 dpf. Note the proper formation of the major axial vessels, dorsal aorta (DA), and posterior cardinal vein (PCV), as well as the inter-segmental vessels (ISVs) and the dorsal longitudinal anastomotic vessel (DLAV). **D–I:** Representative embryos treated from 7 hpf to 2 dpf with SMI496 at the indicated concentrations. Note the abnormal and stunted formation of the intersegmental vessels (arrows in **F** and **I**) and the absence of the dorsal longitudinal anastomotic vessel. **J–L:** Representative images of partial recovery after removal of SMI496 from the embryo medium and subsequent growth for an additional 3 days in compound-free water. **M–O:** Representative fluorescent images of live *vegfr2-gfp* transgenic zebrafish at 2 dpf treated with SMI355 at the designated concentrations for 4 hours starting at 7 hpf. Note normal vasculogenesis and angiogenesis. All panels are oriented with rostral to the left and all lateral views are from the left side. Scale bars = $\sim 300 \mu\text{m}$.

a $t_{1/2}$ of ~ 8 hours for control wound closure compared with a $t_{1/2}$ of ~ 24 hours for the SMI496-treated cultures ($P < 0.001$) (Figure 5H).

SMI496 Disrupts Zebrafish Developmental Angiogenesis

There is a significant degree of conservation between the $\beta 1$ I-like domains of the zebrafish and mammalian integrins.³⁸ We therefore predicted a similar interaction between zebrafish integrin $\beta 1$ I-like domain and SMI496. Accordingly, we studied the effect of SMI496 on angiogenesis *in vivo* using zebrafish. To monitor vascular development *in vivo* and over real time, we used a vascular transgenic zebrafish expressing green fluorescent protein under the control of the vascular endothelial growth factor receptor 2 promoter (*vegfr2-gfp*).³³ In contrast with the axial vessels (dorsal aorta and posterior cardinal vein), which form by vasculogenesis, the intersegmental vessels (ISVs), dorsal longitudinal anastomotic vessels, and parachordal vessels develop through angiogenesis.^{39–41} Embryos treated with 4 or 20 $\mu\text{mol/L}$ SMI496 developed normally but showed significant and dose-dependent suppression of ISV development compared with those treated with vehicle alone (Figure 6, A–I). Often, the endothelial cells failed to migrate along the myosepta from the dorsal aorta ($>80\%$, $n = 160$ in two separate experiments) and followed abnormal paths, failing to interconnect and form the regular lattice of vessels present on either side of the trunk (Figure 6, F and I, arrows). Consequently, the dorsal longitudinal anastomotic vessels were poorly formed or absent in the embryos treated with SMI496. In contrast, the negative con-

trol SMI, SMI355, at 30 $\mu\text{mol/L}$ caused no significant changes in developmental angiogenesis ($n = 80$) (Figure 6, M–O). To determine whether the effects of SMI496 were reversible, zebrafish embryos were treated as before for 4 hours, and then were rinsed and allowed to recover in fresh embryo medium for an additional 3 days. The results indicated significant recovery of the vascular phenotype in $\sim 85\%$ ($n = 80$) of the treated embryos (Figure 6, J and K). However, a minority of the embryos ($\sim 12\%$) showed either incomplete recovery or a significant continuing inhibition of vascular sprouting (Figure 6L). Similar results were seen in the cranial vessels (supplemental Figure S3, see <http://ajp.amipathol.org>). Collectively, these results corroborate the *in vitro* function of SMI496 and further indicate that this small compound acts at a crucial stage of developmental angiogenesis without significantly affecting vasculogenesis.

Characterization of Zebrafish $\alpha 2$ Integrin Subunit

To assess the role of $\alpha 2$ integrin during zebrafish vascular development we began by characterizing *Danio rerio* integrin $\alpha 2$. Our analysis of the available zebrafish genome revealed the presence of a single gene (*itga2*) encoding zebrafish $\alpha 2$ integrin (chromosome 10). We found that the *itga2* gene (gene identification number: 100151619) maintains conserved synteny with the human and mouse integrin $\alpha 2$ genes (supplemental Figure S4A, see <http://ajp.amipathol.org>). By RT-PCR, using *Danio rerio* *itga2*-specific primers, we detected mRNA/expression of *itga2* throughout zebrafish embryogenesis (supplemental Figure S4B, see <http://ajp.amipathol.org>). The ex-

Figure 7. Characterization of the $\alpha 2$ morphant phenotype. **A:** Representative bright-field image of four 1.5 dpf control embryos. **B:** Representative bright-field image of six matched $\alpha 2$ -MO injected embryos. **C:** Control vasculature as visualized by GFP and corresponding to the embryos presented in **A**. **D:** $\alpha 2$ morphant vasculature corresponding to the embryos presented in **B**. Note the defect in trunk and tail angiogenesis. **E:** Summary of the dose-response relationship between morpholino injection amount and overall phenotypic penetrance. Scale bar = 1 mm. $\alpha 2$ knockdown inhibits multiple aspects of developmental angiogenesis. **F–I:** Fluorescent micrographs of live *vegfr2-gfp* zebrafish embryos focusing on the vasculature at 1.5 dpf of control and integrin $\alpha 2$ morphant embryos. **F:** Representative control trunk to tail region exhibiting complete formation of the axial vessels, ISVs and caudal venous plexus (CV). **G** and **H:** morphant trunk regions exhibiting abnormal ISV development. **I:** Representative morphant tail region exhibiting abnormal CV development. Scale bars = ~200 μ m.

pression pattern indicates maternal contribution of $\alpha 2$ at early time points (<3 hours postfertilization [hpf]) and the initiation and increase of zygotic expression over time (supplemental Figure S4B, see <http://ajp.amipathol.org>). Our results are supported by zebrafish *itga2* gene expression profiling data (supplemental Figure S4C, see <http://ajp.amipathol.org>).⁴² The predicted zebrafish integrin $\alpha 2$ protein exhibit overall 49 and 47% identities with the human and mouse counterparts, respectively (supplemental Figure S5, see <http://ajp.amipathol.org>). Comparison of the $\alpha 2$ I domain alone revealed 57% identity between fish, human, and mouse species (un-

derlined regions, supplemental Figure S5, see <http://ajp.amipathol.org>).

Zebrafish $\alpha 2$ Integrin and Vascular Development

To examine the role of $\alpha 2$ integrin during blood vessel formation *in vivo* we applied a morpholino knockdown approach. We designed a translation blocking morpholino targeting the 5'-untranslated region and translation start of zebrafish *itga2*. Microinjection of 1 to 10 ng of morpholino ($\alpha 2$ -MO) was capable of inducing a similar and dose-dependent phenotype (Figure 7, A–E). The $\alpha 2$ morphant phenotype was characterized by generalized twisting of the body associated with a curly tail (Figure 7B). We found similar results using the vascular transgenic and wild-type embryos. We eliminated morpholino off-target effects due to p53 activation by co-knockdown with p53-MO (supplemental Figure S6, see <http://ajp.amipathol.org>). We verified $\alpha 2$ knockdown by assessing the total $\alpha 2$ protein levels of the morphant via whole-mount immunostaining (supplemental Figure S7, see <http://ajp.amipathol.org>). Consistent with the SMI496 treatment, analysis of 1.5 dpf $\alpha 2$ morphant embryo vascular development revealed substantial inhibition of developmental angiogenesis (Figure 7). We found a significant defect in ISV development associated with a total decrease in the branching morphogenesis of the caudal venous plexus (Figure 7, F–I). A closer examination revealed that these vessels were largely nonfunctional in the absence of $\alpha 2$. Concurrently, by using the *Tg(fli1a:nEGFP)*⁷, a transgenic zebrafish in which endothelial cell nuclei are labeled with a GFP containing the nuclear localization sequence,³⁴ we found a significant reduction in endothelial cell number at the level of the deficient sprouting. These results suggest that a functional integrin $\alpha 2\beta 1$ is also required for endothelial cell proliferation (supplemental Figure S8, see <http://ajp.amipathol.org>). By live differential interference contrast videomicroscopy, we found that the partially formed vessels contained little to no red blood cell circulation (supplemental Videos SV1 to SV3, see <http://ajp.amipathol.org>). Additional analysis of angiogenic subintestinal vessel development at 3 dpf also revealed that the $\alpha 2$ knockdown inhibited subintestinal vessel formation (Figure 8, A–E). Overall, these results indicate a central role for $\alpha 2$ integrin during angiogenic blood vessel formation.

Figure 8. $\alpha 2$ knockdown disrupts angiogenic subintestinal vessel (SIV) development. **A:** Representative control embryo displaying clear formation of the subintestinal vessels by 3 dpf. **B–D:** Matched $\alpha 2$ morphant embryos exhibiting inhibition of subintestinal vessel formation in a dose-dependent manner. All vessels were visualized by endogenous alkaline phosphatase staining. Scale bar = 250 μ m. **E:** Graphic summary of the number of subintestinal vessel plexus intersection points. Data are the mean $\pm$ SD of 10 embryos/dose from two independent experiments.

Discussion

The $\alpha 2\beta 1$ integrin plays key roles in hemostasis and endothelial cell biology; yet, few inhibitors of the receptor have been identified. Inhibitors include function blocking antibodies^{3,9,43} and aromatic polyketides from *Streptomyces*, proposed to bind the closed conformation of the $\alpha 2$ domain, coordinating with the magnesium ion and neighboring residues of the MIDAS adhesion site.⁴⁴ These compounds block $\alpha 2$ domain-collagen binding, and the adhesion of integrin-expressing Chinese hamster ovary cells to type I collagen. The snake venom protein rhodocetin binds to the $\alpha 3$ - $\alpha 4$ loop and adjacent α helices in the $\alpha 2\beta 1$ integrin-collagen binding site and blocks integrin-collagen interactions.^{45,46} The C-lectin type snake venom protein EMS16 binds the $\alpha 2$ domain, blocking collagen-induced platelet aggregation and endothelial cell migration in collagen.⁴⁷ Saratin, from the leech, also disrupts platelet $\alpha 2$ domain-collagen interactions.⁴⁸ As alternatives to these natural products, families of small molecule inhibitors of integrin function have recently been developed.^{30,44} Those used in the present study target $\alpha 2\beta 1$ integrin I domain function and are patterned after peptide-mimetic inhibitors of other integrins. These compounds have greater potencies and specificities than inhibitors of this integrin studied previously.³⁰

To assay the effects of the SMLs on *in vitro* angiogenesis, we used a method replicating capillary tube formation, the last stage of angiogenesis. Thus, confluent endothelial cell monolayers cultured in a serum-free defined medium, including fibroblast growth factor-2 and VEGF₁₆₅⁹ and overlaid with a collagen gel, form capillary-like tubes within 12 to 24 hours. In this assay SMI496, which binds between the I domains of $\beta 1$ and $\alpha 2$ subunits,³² inhibited capillary tube formation by approximately 60 to 70% whereas the negative control compounds had very little effect. Notably, the level of inhibition of angiogenesis by SMI496 was nearly identical to that exhibited by an anti- $\alpha 2\beta 1$ integrin function blocking antibody, or triple helical peptides containing the integrin-binding GFPGER₅₀₂₋₅₀₇ sequence.⁹ It is possible that incomplete inhibition of angiogenesis occurs, because another integrin or class of receptors besides the $\alpha 2\beta 1$ integrin may contribute to collagen-induced angiogenesis. However, contributions of the $\alpha 1\beta 1$ and $\alpha v\beta 3$ integrins to collagen-induced angiogenesis have been ruled out in this system.⁹

In cell proliferation studies, SMI496 either failed to affect endothelial cell growth for up to 3 days in culture or exhibited minor inhibitory effects at later times. The fact that SMI496 showed no evidence of toxicity and was active in our rapid serum-free angiogenesis assay in which cell proliferation probably plays little, if any, role,⁹ suggests that the slight activity of SMI496 on cell growth may not be mechanistically related to its anti-angiogenic activity.

The effects of SMLs on endothelial cell-collagen interactions were next examined using flow cytometry. Endothelial cell binding to collagen was markedly inhibited by SMI496 or an anti- $\alpha 2\beta 1$ integrin function blocking anti-

body. Moreover, SMI496 disrupted endothelial cell interactions with substrate-bound collagens, as evidenced by reduced cell-substratum interactions accompanied by an apparent collapse of the actin cytoskeleton. These data imply that SMI496 may disrupt endothelial cell- $\alpha 2\beta 1$ integrin-collagen ligation and perhaps even displace $\alpha 2\beta 1$ integrins already bound to collagen. Notably, the activity of SMI496 is reminiscent of that of endorepellin, a fragment of perlecan, which binds to the $\alpha 2\beta 1$ integrin and disrupts its function.^{16,21} Lastly, endothelial cell migration on collagen was delayed by SMI496, even in the presence of a growth inhibitor. Thus, SMI496 inhibits endothelial cell collagen binding, migration on collagen, and collagen-induced capillary morphogenesis, but with little effect on proliferation.

To examine the activities of SMI496 on *in vivo* angiogenesis we used the zebrafish, owing to their rapid embryonic development and their transparency, permitting direct *in vivo* observation of vascular development. In addition, the zebrafish model is amenable to the testing of small molecules⁴⁹⁻⁵² and screening of angiogenic compounds supplemented to the embryo media.^{33,53} To monitor vascular development, we used the vascular *vegfr2-gfp* transgenic zebrafish harboring endothelial-specific expression of GFP.³³ Embryos treated with 4 to 30 μ mol/L SMI496 developed normally, but showed significant and dose-dependent suppression of ISVs compared with vehicle alone. Our results indicate that the biological effects of SMI496 are partially reversible and very specific for sprouting blood vessels for several reasons. First, the development of the major axial vessels was not altered, and therefore it seems that SMI496 does not affect vasculogenesis. Second, on removal of SMI496, most of the embryos recovered to near normality, indicating that the endothelial cells in the dorsal aorta and parachordal vessel are still capable of migrating and forming fully functional blood vessels. Our results further indicate there is a precise window of time during which SMI496 exerts its biological activity, because little or no suppression of angiogenesis was observed when treatment began at 1 dpf (data not shown). Notably, by this time the major axial vessels are completely formed, circulation has commenced, and ISVs begin sprouting from the DA. These findings further strengthen the hypothesis that SMI496 might indeed function as an inhibitor of the $\alpha 2\beta 1$ integrin. Therefore, these findings demonstrate an *in vivo* role for the $\alpha 2\beta 1$ integrin in regulating trunk and cranial angiogenesis and suggest that there is a specific window in development during which this integrin is required for proper sprouting angiogenesis, where endothelial cells need to migrate and anastomose to form functional arteriovenous plexuses.

Our analysis of the zebrafish integrin $\alpha 2$ subunit revealed a number of significant points. First, gene expression profile data revealed maternally derived *itga2* expression (<3 hpf). These data suggested the importance of $\alpha 2$ during early embryonic development and clearly indicated the onset of zygotic transcriptional activity at later time points. Second, analysis of the integrin $\alpha 2$ amino acid sequence indicated an evolutionarily conserved $\alpha 2$ within zebrafish and an especially well-con-

served I domain. Third, morpholino knockdown of $\alpha 2$ implied a central requirement for embryo viability. We have found a significant amount of embryo lethality by 1 dpf, suggesting that $\alpha 2$ is required for proper development. We believe only embryos exhibiting a partial knockdown are therefore able to survive at later time points. Accordingly, our whole-mount immunostaining revealed partial knockdown because certain portions of the embryo body were nearly devoid of positive staining, whereas other regions remained reactive. Finally, our analysis of vascular development in $\alpha 2$ integrin morphant embryos supported a central role for $\alpha 2$ during sprouting angiogenesis in the trunk and tail and branching morphogenesis of the caudal vein. In addition, $\alpha 2$ morphants exhibited a significant decrease in endothelial cell number, suggesting an important role for $\alpha 2$ integrin in endothelial cell proliferation. These results are the first to implicate integrin $\alpha 2$ during developmental angiogenesis.

In summary, we show for the first time that SMI496 inhibits endothelial cell-collagen binding, migration on collagen, and collagen-induced angiogenesis. SMI496 functionally disrupts $\alpha 2\beta 1$ by binding between the I domain of $\alpha 2$ and the I-like domain of $\beta 1$ subunits, thereby allosterically inhibiting $\alpha 2\beta 1$ integrin function. This would be the first report implicating a role for the $\beta 1$ I-like domain in angiogenesis. Although SMI496 may function by disrupting cell-collagen ligation, it remains to be seen whether it also influences $\alpha 2\beta 1$ activation or clustering, two other components of integrin function and signaling. Our results suggest that SMI496 and related compounds may have significant therapeutic potential. Thus, because of their small size, SMIs should be highly diffusible and easily delivered to target tissues. In tissue stroma, SMIs are predicted to arrest angiogenesis when endothelial cells attempt to engage type I collagen fibrils to initiate capillary tube formation. Yet, SMI496 should not affect endothelial cell homeostasis and viability. SMIs are thus attractive candidates as therapies to combat pathological changes including tumor growth and metastasis, in which angiogenesis plays a prominent role. Finally, the discovery that the partial knockdown of integrin $\alpha 2$ causes a phenotype characterized by abnormal and non-functional angiogenic sprouting, both in the trunk and cephalic region, underlines a key functional role for this receptor during developmental angiogenesis.

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