Suppression and Regression of Choroidal Neovascularization by Systemic Administration of an α5β1 Integrin Antagonist

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ABSTRACT
Integrin α5β1 plays an important role in developmental angiogenesis, but its role in various types of pathologic neovascularization has not been completely defined. In this study, we found strong up-regulation of α5β1 in choroidal neovascularization. Implantation of an osmotic pump delivering 1.5 or 10 μg/h (~1.8 or 12 mg/kg/day) of 3-[2-{1-alkyl-5-[pyridin-2-ylamino]-methyl]-pyrrolidin-3-yloxy}-acetylamino)-2-(alkylamino)-propionic acid (JSM6427), a selective α5β1 antagonist, caused significant suppression of choroidal neovascularization; the area of neovascularization was reduced by 33 to 40%. When an osmotic pump delivering 10 μg/h of JSM6427 was implanted 7 days after rupture of Bruch’s membrane, there was terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining in vascular cells within the neovascularization and significant regression of the neovascularization over the next week. JSM6427 also induced apoptosis of cultured vascular endothelial cells. Fibronectin stimulates phosphorylation of extracellular signal-regulated kinase (ERK) in α5β1-expressing cells that is blocked by JSM6427. These data suggest that α5β1 plays a role in the development and maintenance of choroidal neovascularization and provides a target for therapeutic intervention.

Vascular endothelial cells participating in angiogenesis get signals from several sources. Surrounding tissue that senses the need for increased blood (oxygen) delivery is an important source of soluble stimulators. In hypoxic tissue, the transcription factor hypoxia inducible factor-1 (HIF-1) is stabilized, resulting in up-regulation of several genes (Manalo et al., 2005). Vascular endothelial growth factor (VEGF) is an important hypoxia-regulated soluble signal produced by surrounding tissue that promotes neovascularization. Things other than hypoxia, such as cytokines, also up-regulate VEGF, and in many instances, this seems to be through HIF-1 or HIF-2 (Bardos et al., 2004; Haddad and Harb, 2005). Although other soluble signals undoubtedly play a role, it is clear that inhibition of VEGF is a useful strategy for treatment of neovascular diseases (Ribbati, 2005).

Another source of signals is the extracellular matrix (ECM). The ECM seems capable of providing both stabilizing signals that suppress neovascularization and stimulatory signals (Sottile, 2004). Integrins are cell surface receptors that mediate signaling from the ECM, and signaling from ECM is modulated by alterations in the integrin population on the cell surface. Therefore, it is important to determine in any given situation which integrins mediate stimulation of neovascularization and which seem to suppress it.

Integrins α3β3 and α5β3 are up-regulated on endothelial cells participating in several types of neovascularization, including tumor vessels and retinal neovascularization (Brooks et al., 1994a,b; Luna et al., 1996). Antagonists of α3β3 and α5β3 suppress tumor neovascularization and retinal neovascularization (Hammes et al., 1996; Luna et al., 1996). However, the same antagonists of α3β3 and α5β3 that suppress retinal neovascularization, have no identifiable suppressive effect on choroidal neovascularization in mice (P.A. Campochiaro, unpublished data). Gene knockout studies have shown that deletion of α5, β3, and/or β5 fails to block developmental angiogenesis and in some cases may enhance an-
giogenesis (Hynes, 2002). Therefore, it seems that the role of integrins in neovascularization in one situation does not guarantee participation in a different vascular bed and different disease process.

Integrin $\alpha_\beta_1$ is also up-regulated on activated endothelial cells and tumor blood vessels (Collo and Pepper, 1999; Kim et al., 2000; Magnusson et al., 2005; Parsons-Wingerter et al., 2005). Antagonists of $\alpha_\beta_1$ suppress angiogenesis on chick chorioallantoic membrane induced by FGF2, tumor necrosis factor-$\alpha$ or interleukin-8 and in murine tumor models (Kim et al., 2000; Stoeltzing et al., 2003; Magnusson et al., 2005). Integrin $\alpha_\beta_1$ also plays a critical role in developmental angiogenesis (Yang et al., 1993; Hynes, 2002). During vascularization of the central nervous system, angiogenic sprouts express high levels of $\alpha_\beta_1$, and it is markedly reduced as the vessels mature (Milner and Campbell, 2002). Thus, $\alpha_\beta_1$ seems to promote angiogenesis in multiple settings, but, as noted above, one cannot assume that it is proangiogenic in all tissues and pathologies. In this study, we have explored the role of $\alpha_\beta_1$ in choroidal neovascularization, the most common cause of severe vision loss in elderly Americans (Klein et al., 1993).

Materials and Methods

Mouse Model of Choroidal Neovascularization. Mice were treated in accordance with the Association for Research in Vision and Ophthalmology guidelines for the use of animals in research. Choroidal neovascularization was induced by laser photocoagulation-induced rupture of Bruch’s membrane as described previously (Tobe et al., 1998). In brief, 5- to 6-week-old female C57BL/6J mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight), and pupils were dilated with 1% tropicamide. Three burns of 532 nm diode laser photocoagulation (75-μm spot size, 0.1-s duration, 120 mW) were delivered to each retina with the slit lamp delivery system of an OcuLight GL diode laser (Iridex, Mountain View, CA) using a handheld cover slip as a contact lens to view the retina. Burns were performed in the 9, 12, and 3 o’clock positions of the posterior pole of the retina. Production of a bubble at the time of laser, which indicates rupture of Bruch’s membrane, is an important factor in obtaining choroidal neovascularization; therefore, only burns in which a bubble was produced were included in the study.

Immunohistochemistry and Histochemistry. Two weeks after rupture of Bruch’s membrane, mice were euthanized and eyes were removed and fixed for 30 min in 0.1 M phosphate buffer, pH 7.6, containing 4% paraformaldehyde and 5% sucrose. After 30 min, corneas and lenses were removed and then fixation was continued for another hour. After washing overnight with 0.1 M phosphate buffer containing 20% sucrose, the eyecups were frozen in optimum cutting temperature embedding compound (Miles Diagnostics, Elkhart, IN). Ocular frozen sections (10 μm) were dried with cold air for 20 min, fixed in freshly prepared 4% paraformaldehyde in 0.05 M phosphate-buffered saline (PBS) at room temperature for 15 min, and rinsed with 0.05 M Tris-buffered saline (TBS) for 10 min. Endogenous peroxidases were inhibited by a 15-min incubation with 0.75% H$_2$O$_2$ in methanol. Sections were washed three times in 0.05 M TBS and nonspecific binding sites were blocked by incubating in 10% normal goat serum in 0.05 M TBS overnight. For controls, nonimmune IgG (Vector Laboratories, Burlingame, CA) was substituted for primary antibody. After two rinses with TBS, sections were incubated for 30 min at room temperature with secondary antibody,

![Fig. 1. Immunohistochemical staining for integrin $\alpha_5$ subunit in mice with choroidal neovascularization. Adult C57BL/6J mice had laser-induced rupture of Bruch’s membrane in each eye. Two weeks after laser treatment, mice were euthanized, eyes were removed, and frozen sections were cut through rupture sites. Some sections were stained with Grifonia simplicifolia, which selectively stains vascular cells and allows visualization of choroidal neovascularization (A). Adjacent sections were immunohistochemically stained for integrin $\alpha_5$ subunit (B). Superimposed image from a DAPI-stained section shows the retinal cells in the ONL, inner nuclear layer (INL), and ganglion cell layer (GCL), confirming that the cells expressing $\alpha_5$ integrin are in the subretinal space (C). Retina and choroid remote from Bruch’s membrane rupture sites showed staining of retinal vessels with GSA, but no choroidal neovascularization (D) and no staining for integrin $\alpha_5$ subunit (E and F). Scale bar, 100 μm.](https://image.com/1821)
1:1000 fluorescein isothiocyanate-conjugated goat anti-rabbit IgG F(ab’)2 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Sections were counterstained with DAPI (Kirkgaard and Perry Laboratories, Gaithersburg, MD) and mounted with Aquamount (British Drug House, Poole, Dorset, UK).

Serial sections were stained with biotinylated *Griffonia simplicifolia* lectin B4 (GSA; Vector Laboratories) to identify choroidal neovascularization as described previously (Ozaki et al., 1998). In brief, slides were incubated in methanol/H$_2$O$_2$ for 10 min at 4°C, washed with 0.05 M TBS, pH 7.6, and incubated for 50 min in 10% normal porcine serum. Slides were incubated for 2 h at room temperature with biotinylated GSA. After washing, the slides were incubated in streptavidin-phosphatase and developed with HistoMark Red (Kirkgaard and Perry Laboratories) according to the manufacturer’s instructions. Sections were dehydrated and mounted with Cytoseal. Stained sections were examined with a Nikon microscope and captured as digital files with a Nikon digital still camera (DXM1200; Nikon Instruments Inc., New York, NY).

**Systemic Administration of JSM6427 in Mice with Rupture of Bruch’s Membrane.** Two different concentrations of JSM6427, 3 mg/ml in PBS and 20 mg/ml in 100 mM glycine/NaOH, pH 4.0, or the corresponding vehicle alone were loaded into osmotic mini-pumps (model 2002; Alza Corp., Palo Alto, CA) with internal volume of 200 µl and mean pumping rate of 0.5 µl/h. Pumps were implanted beneath the skin of the back and the following day the mice had laser-induced rupture of Bruch’s membrane at 3 locations in each eye. After 14 days, the mice were perfused with 1 ml of PBS containing 50 mg/ml fluorescein-labeled dextran (2 × 10$^6$ average molecular weight) and stained with biotinylated GSA lectin B4 (GSA; Vector Laboratories) to identify choroidal neovascularization. Sections were dehydrated and mounted with Cytoseal. Stained sections were examined with a Nikon microscope and captured as digital files with a Nikon digital still camera (DXM1200; Nikon Instruments Inc., New York, NY).

**Fig. 2.** Systemic delivery of JSM6427 by osmotic minipump suppresses the development of choroidal neovascularization at Bruch’s membrane rupture sites. A, the osmotic minipumps were approximately 30 mm long. B, the implanted minipumps were visible as humps (arrows) beneath the skin of the back. Mice implanted with pumps containing 3 mg/ml JSM6427 received approximately 1.5 µg/h JSM6427 and choroidal flat mounts after perfusion with fluorescein-labeled dextran showed small areas of choroidal neovascularization at rupture sites (C, arrows) compared with areas of choroidal neovascularization seen in mice implanted with vehicle (D, arrows). Image analysis confirmed that there was significantly less choroidal neovascularization in mice that received 1.5 µg/h JSM6427 compared with those that received vehicle (E). Mice implanted with pumps containing 20 mg/ml JSM6427 received approximately 10 µg/h JSM6427 and also seemed to have smaller areas of choroidal neovascularization (F, arrows) than mice that received vehicle (G, arrows). Image analysis showed a statistically significant difference from vehicle (H) in the same range as that seen after infusion of 1.5 µg/h of JSM6427∗, p = 0.0005; †, p = 6 × 10$^{-8}$ by Mann-Whitney U test. Scale bar, 100 µm.
weight; Sigma-Aldrich, St. Louis, MO) and choroidal flat mounts were prepared as described previously. In brief, eyes were removed and fixed for 1 h in 10% phosphate-buffered formalin. The cornea, lens, and retina were removed, and four radial cuts were made in the eyecup, allowing it to be flat-mounted in aqueous mounting medium. Flat mounts were examined by fluorescence microscopy, and images were digitized using a three-color charge-coupled device video camera and a frame grabber. Image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD) was used to measure the total area of choroidal neovascularization at each rupture site.

To assess the effect of JSM6427 on established choroidal neovascularization, mice had rupture of Bruch’s membrane at three locations in each eye. After 7 days, 9 mice were perfused with fluorescein-labeled dextran, and the baseline area of neovascularization at each rupture site was measured. The remaining mice had implantation of osmotic minipumps containing 20 mg/ml JSM6427 in 100 mM sodium phosphate buffer, 50 mM NaCl, pH 7.4, or vehicle alone. At 7 days after implantation, some mice were euthanized for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). The rest of the mice were perfused with fluorescein-labeled dextran 7 days after implantation, and the area of choroidal neovascularization at rupture sites was measured.

Intravitreous Injections of JSM6427. Mice had rupture of Bruch’s membrane at three locations in each eye and were given an intravitreous injection of 1 μl of vehicle (PBS or 100 mM phosphate buffer, and 50 mM NaCl, pH 7.4) containing of 3 or 20 μg of JSM6427 in one eye and 1 μl of vehicle alone in the other eye on days 0 and 7. For an additional control, some mice received no injection. On day 14, mice were perfused with fluorescein-labeled dextran, and the area of choroidal neovascularization at Bruch’s membrane rupture sites was measured.

Identification of Apoptotic Cells in Vivo by TUNEL. Eyes were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and frozen in OCT. Serial 10-μm sections were cut through each rupture site. Sections from day 7 baseline eyes (D), a consequence of the laser treatment 7 days before. Sections from day 14 eyes that had been treated with JSM6427 also showed apoptotic cells in the ONL, but in addition there were yellow cells within choroidal neovascularization lesions (E and G, arrows) as a result of colocalization of TUNEL and GSA, indicating apoptosis of cells with the choroidal neovascularization. Sections from eye treated with vehicle showed apoptotic cells in the ONL, but not within the choroidal neovascularization (F). Measurement of the area of choroidal neovascularization by image analysis confirmed that there was a significant reduction in mice treated with JSM6427 compared with the amount seen at baseline or in mice treated with vehicle (H). *, p = 0.0039 by linear mixed model for comparison with baseline; †, p = 0.0283 by linear mixed model for comparison with vehicle. P values were adjusted for multiple comparisons using Dunnett’s method. Scale bars: A–F, 100 μm; G, 50 μm.

**Fig. 3.** Systemic delivery of JSM6427 by osmotic minipump causes regression of choroidal neovascularization. Adult C57BL/6J mice had laser-induced rupture of Bruch’s membrane at three locations in each eye. Seven days after laser treatment, nine mice were perfused with fluorescein-labeled dextran, and the baseline amount of choroidal neovascularization at 7 days (A, arrows) was measured by image analysis. The remainder of the mice had implantation of osmotic minipumps containing 20 mg/ml JSM6427 in 100 mM sodium phosphate buffer, 50 mM NaCl, pH 7.4, or vehicle alone. At 7 days after implantation, some mice were euthanized for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). The rest of the mice were perfused with fluorescein-labeled dextran 7 days after implantation, and the area of choroidal neovascularization at rupture sites was measured.
site. Sections were fixed with 1% paraformaldehyde for 10 min at room temperature, and TUNEL was done with the ApopTag Red Kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions. The sections were also histochemically stained with GSA as described above. Slides were also stained with DAPI and mounted in aqueous mounting medium.

**Statistical Analysis.** Data were analyzed using a linear mixed model accounting for possible correlations in measurements from the same mice. Dunnett's adjustment was made for multiple comparisons.

**Solid Phase Binding Assay.** The inhibiting activity and integrin selectivity of the integrin inhibitor was determined in a solid phase binding assay using soluble integrins and coated extracellular matrix protein. Binding of integrins was then detected by specific antibodies in an enzyme-linked immunosorbent assay. Fibronectin and vitronectin were purchased from Sigma (St Louis, MO). The integrin \( \alpha_5\beta_1 \) extracellular domain Fc-fusion protein was a generous gift from M. Humphries (University of Manchester), and \( \alpha_5\beta_3 \) was purchased from Chemicon (Chemicon Europe, Germany). The integrin antibodies were purchased from Pharmingen, BD Bioscience Europe (\( \alpha_5\beta_2 \), \( \alpha_5\beta_3 \), and GSA (anti-human-Fc-HRP antibody conjugate and anti-human-Fc-HRP antibody conjugate and anti-mouse-HRP conjugate).

The detection of HRP was performed using HRP substrate solution 3.3.3.5.-tetramethylbiphenylenediamine (Serumun Diagnostica GmbH, Dolgenbrodt, Germany) and 1 M H₂SO₄ for stopping the reaction. The developed color was measured at 450 nm.

\( \alpha_5\beta_2 \)-Nunc Immuno maxisorb plates (Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with fibronectin (0.25 \( \mu \)g/ml) in 15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6. All subsequent washing and binding were performed in 25 mM Tris, pH 7.6, 150 mM NaCl, 1 mM MnCl₂, and 1 mg/ml BSA. The plates were blocked with 3% BSA in PBS 0.1% Tween 20 for 1 h at room temperature. Soluble integrin \( \alpha_5\beta_2 \) (0.5 \( \mu \)g/ml) and a serial dilution of integrin inhibitor were incubated in the coated wells for 1 h at room temperature. The detection antibody (anti-human-Fc-HRP antibody conjugate) was then applied for 1 h at room temperature, and the binding was visualized as described above. For the \( \alpha_5\beta_2 \) assay, plates were coated with vitronectin (1 \( \mu \)g/ml) and blocked as described for \( \alpha_5\beta_1 \). Soluble \( \alpha_5\beta_3 \) (1 \( \mu \)g/ml) was incubated with a serial dilution of integrin inhibitor for 1 h at room temperature. Primary (anti-\( \alpha_5\beta_3 \)) and secondary antibody (anti-mouse-HRP conjugate) were applied for 1 h at RT, and the binding was visualized as described above. All IC₅₀ measurements were performed at least 30 times.

**Identification of Apoptotic Cells in Vitro by TUNEL and Fluorescent Activated Cell Sorting.** Human umbilical vein endothelial cells were maintained in endothelial cell growth medium (PromoCell, Heidelberg, Germany) and grown to 80% confluence, then harvested, washed, and placed in six-well plates coated with 100 \( \mu \)g/ml fibronectin or 0.002% poly-L-lysine (Sigma-Aldrich, Munich, Germany) and 1 M H₂SO₄ for stopping the reaction. The developed color was measured at 450 nm.

**Results**

**Expression of Integrin \( \alpha_5 \) Subunit Is Increased in Vascular Cells Participating in Choroidal Neovascularization.** Two weeks after laser-induced rupture of Bruch's membrane staining with GSA showed large choroidal neovascularization lesions at rupture sites (Fig. 1A, arrows). Adjacent sections immunohistochemically stained for integrin \( \alpha_5 \) subunit showed strong labeling throughout the entire choroidal neovascularization lesion (Fig. 1B, arrows). Superimposed images from DAPI-stained sections showed the overlying neural retina, confirming that the cells labeled with anti-\( \alpha_5 \) are in the subretinal space (Fig. 1C). Regions of retina remote from Bruch's membrane rupture sites showed no choroidal neovascularization and no staining for \( \alpha_5 \) subunit (Fig. 1, D–F). This suggests that expression of \( \alpha_5 \) subunit in vascular cells in the eye under normal circumstances is below the level of our staining technique to detect it, but there is strong up-regulation of \( \alpha_5 \) subunit expression in vascular cells participating in choroidal neovascularization.

**JSM6427 Is a Selective Antagonist of Integrin \( \alpha_5\beta_1 \).** The integrin inhibitor JSM6427 is an antagonist of binding to integrin \( \alpha_5\beta_1 \) and is at least 1200-fold less potent in the inhibition of binding to \( \alpha_5\beta_3 \), the integrin that is most closely

![Fig. 4. JSM6427 induces apoptosis in cultured vascular endothelial cells. Human umbilical vein endothelial cells were grown in fibronectin coated dishes for 48 h in serum-free medium containing various concentrations of JSM6427 or camptothecin. TUNEL-stained cells were detected by fluorescence-activated cell sorting. Each bar shows the mean (± S.D.) percentage of apoptotic cells per culture generated from one experiment performed in triplicate. * p < 0.01 by ANOVA with Dunnett's correction for multiple comparisons.](https://example.com/fig4.png)
related to \( \alpha_5 \beta_1 \) in structure. The selectivity against other integrins such as \( \alpha_5 \beta_3 \) and \( \alpha_{10} \beta_3 \) is significantly higher, 3700-fold or \( >100,000 \)-fold, respectively (data not shown).

**Subcutaneous Delivery of JSM6427 Suppresses Choroidal Neovascularization.** Osmotic minipumps (Fig. 2A) that release 1.5 or 10 \( \mu \)g/h JSM6427 or vehicle alone were implanted beneath the skin on the backs of mice (Fig. 2B), and the following day, Bruch’s membrane was ruptured in three locations in each eye. Fourteen days after rupture of Bruch’s membrane, the size of choroidal neovascularization lesions at rupture sites in mice that received 1.5 or 10 \( \mu \)g/h JSM6427 (Fig. 2, C and F) appeared smaller than corresponding control mice that received infusion of vehicle (Fig. 2, D and G). Measurement of the size of the lesions by image analysis confirmed that for both infusion rates of JSM6427, 1.5 (Fig. 2E) and 10 \( \mu \)g/h (Fig. 2H), choroidal neovascularization lesions were significantly smaller than those in corresponding vehicle-treated control mice by approximately 33 and 40%, respectively.

**Subcutaneous Delivery of JSM6427 Causes Regression of Established Choroidal Neovascularization.** Mice had laser-induced rupture of Bruch’s membrane, and after 7 days, some mice were used to measure the baseline amount of choroidal neovascularization (Fig. 3A). In the remainder of the mice, an osmotic minipump was implanted that released 10 \( \mu \)g/h JSM6427 or vehicle. After 7 days of treatment, the mice infused with JSM6427 had small choroidal neovascularization lesions at Bruch’s membrane rupture sites (Fig. 3B), whereas mice infused with vehicle had lesions that appeared larger (Fig. 3C). TUNEL showed labeling within the outer nuclear layer (ONL) of the retina overlying choroidal neovascularization in day 7 baseline eyes (Fig. 3D). This was probably a consequence of the laser photocoagulation that was used to induce rupture of Bruch’s membrane, although it is possible that the presence of choroidal neovascularization is damaging to the overlying retina as a result of interference with transmission of oxygen from the choroid, interference with transmission of survival signals from the retinal pigmented epithelium, or some other reason. At 14 days after rupture of Bruch’s membrane, mice infused with JSM6427 for the previous 7 days (Fig. 3E) and those infused with vehicle (Fig. 3F) showed apoptosis in the retina overlying choroidal neovascularization, but only the mice infused with JSM6427 showed apoptosis of vascular cells within the choroidal neovascularization as expected for the proposed mechanism of action and confirmed with in vitro apoptosis assays (Fig. 4). Figure 3G shows the boxed region in Fig. 3E at high magnification, providing better visualization of two yellow cells (arrows) because of colocalization of GSA and ApopTag red. Measurement of the size of choroidal neovascularization lesions by image analysis showed that mice infused with JSM6427 had significantly smaller lesions than those seen at baseline or those seen in mice infused with vehicle (Fig. 3H). This indicates that continuous systemic

![Fig. 5. JSM6427 reduces fibronectin-induced phosphorylation of ERK.](https://doi.org/10.1094/molpharm-04-17-0249-g005)
infusion of JSM6427 causes regression of choroidal neovascularization.

**JSM6427 Causes Apoptosis of Vascular Endothelial Cells in Vitro.** Human umbilical vein endothelial cells were grown in fibronectin-coated wells for 48 h in serum-free medium containing various concentrations of JSM6427. Camptothecin, a strong inducer of apoptosis, was used as a positive control. JSM6427 caused a dose-dependent increase in the percentage of TUNEL-positive cells in cultures ranging from 44% for those incubated with 50 μM to 6% for those incubated in 100 nM (Fig. 4). In cultures incubated in serum-free medium, 5% of cells were TUNEL-positive and 82% of cells were labeled in cultures incubated with 10 μM camptothecin. These data show that JSM6427 induces apoptosis of vascular endothelial cells.

**JSM6427 Reduces Fibronectin-Induced Phosphorylation of ERK.** The mechanism by which ECM components generate survival signals through integrins involves increased phosphorylation of intracellular messengers that increase expression of apoptosis inhibitors such as Bcl-2 (Lee and Ruoslahti, 2005). Phosphorylation of ERK plays a central role in fibronectin-mediated survival signaling through α5β1 in brain capillary endothelial cells (Wang and Milner, 2006). Therefore, we investigated the effect of JSM6427 on fibronectin-induced phosphorylation of ERK in ARPE19 cells, which express α5β1. Serum-starved ARPE19 cells were preincubated with JSM6427 for 10 min in suspension and plated for 15 min on fibronectin, poly-lysine, or kept in suspension. Western blots showed that for cells not preincubated in JSM6427, those plated on fibronectin had a much stronger signal for phosphorylated ERK than those plated on poly-lysine or those kept in suspension (Fig. 5A). Preincubation with JSM6427 before plating on fibronectin caused a dose-dependent reduction in the amount of phosphorylated ERK in the cultures. Quantification of the signal for phosphorylated ERK normalized to the total amount of ERK showed that preincubation with 10 μM JSM6427 completely eliminated the fibronectin-induced stimulation of ERK phosphorylation (Fig. 5B), and the IC50 was 0.23 μM (Fig. 5C).

**Two Intravitreous Injections of JSM6427 Did Not Cause Significant Suppression of Choroidal Neovascularization.** To explore the feasibility of local delivery of JSM6427, mice had laser-induced rupture of Bruch’s membrane followed by intravitreous injection of vehicle or vehicle containing 3 or 20 μg of JSM6427 immediately after laser treatment and 7 days later. Fourteen days after laser treatment, the area of choroidal neovascularization was measured by image analysis. The size of choroidal neovascularization lesions appeared somewhat smaller in eyes treated with JSM6427, but there was not a statistically significant difference compared with vehicle-treated or untreated eyes (Fig. 6). This suggests that weekly intravitreous injections in mice are not able to maintain levels of JSM6427 sufficient to significantly inhibit choroidal neovascularization.

**Discussion**

In this study, we have shown that α5β1 integrin is strongly up-regulated in choroidal neovascularization. This marked differential in expression between vascular cells participating in choroidal neovascularization and vascular cells in established choroidal vessels, suggests that α5β1 may play an important role in growth and maintenance of the new vessels, and blocking α5β1 may provide a way to selectively target choroidal neovascularization. Using a selective antagonist of α5β1, JSM6427, we found this to be the case. Sustained systemic delivery of JSM6427 using an osmotic minipump suppressed the de-

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**Fig. 6.** Two intravitreous injections of JSM6427 failed to suppress choroidal neovascularization at Bruch’s membrane rupture sites. Adult C57BL/6J mice had rupture of Bruch’s membrane at three locations in each eye. On days 0 and 7 after laser treatment, mice were given an intravitreous injection of 3 or 20 μg of JSM6427 in one eye and vehicle in the fellow eye. At day 14, mice were perfused with fluorescein-labeled dextran and the area of choroidal neovascularization at Bruch’s membrane rupture sites was measured by image analysis. There was no statistically significant difference in the size of choroidal neovascularization lesions in eyes injected with 3 (A) or 20 μg (B) of JSM6427 compared with either fellow eyes treated with vehicle or uninjected eyes.
development of choroidal neovascularization, and when infusion was started after the neovascularization was established, it caused it to regress. TUNEL staining showed selective apoptosis in vascular cells participating in the neovascularization in JSM6427-treated mice. This suggests that signaling through αβ1 provides a critical survival signal to endothelial cells in choroidal new vessels that, when blocked, triggers apoptosis. Endothelial cells in established vessels must develop alternative survival signal pathways that eliminate the dependence on αβ1 signaling manifested by endothelial cells in choroidal neovascular lesions. Such a switch has been noted when cerebral vessels mature and change from an angiogenic to a mature phenotype; they stop expressing αβ2, and begin to express αβ2 and αβ1 (Milner and Campbell, 2002). The exquisite selectivity for JSM6427-induced apoptosis for endothelial cells in choroidal neovascularization compared with mature choroidal vessels predicts a good safety profile.

Cell signaling through which integrins promotes cell survival is complex (for review, see Ganciotti and Ruoslahti, 1999). Binding of most integrins to a ligand in the ECM activates focal adhesion kinase, which plays a role in most integrin-mediated effects; survival signaling occurs by activation of focal adhesion kinase along with other signaling molecules that act together to increase levels of Bel-2 or other inhibitors of apoptosis (Lee and Ruoslahti, 2005). Phosphorylation of ERK plays a central role in stimulation of brain capillary endothelial cell survival by fibronectin through αβ1 (Wang and Milner, 2006). JSM6427 blocks fibronectin-induced phosphorylation of ERK, which probably plays a role in its induction of apoptosis.

Despite the apparent safety of sustained systemic administration of JSM6427, local administration has the theoretical benefit of limiting exposure to the rest of the body and reducing the total amount of JSM6427 that is required for treatment. Two intravitreal injections of JSM6427 over the course of 2 weeks failed to suppress choroidal neovascularization. A possible explanation is that sustained exposure of JSM6427 to endothelial cells in choroidal neovascularization is necessary to perturb survival signals, and even brief lapses in blockade of αβ1 signaling may be sufficient to prevent apoptosis. In the future, it will be worthwhile to test this hypothesis by assessing the effect of sustained local delivery of JSM6427.

Choroidal neovascularization occurs in diseases of the retinal pigmented epithelium/Bruch's membrane complex. The most common of these is age-related macular degeneration, which is the most prevalent cause of severe vision loss in patients over the age of 60 in developed countries (Klein et al., 1993). Studies in animal models have demonstrated that VEGF is an important stimulus and that VEGF antagonists suppress the neovascularization (Kwak et al., 2000; Kryzstolik et al., 2002; Saishin et al., 2003). The role of VEGF in patients with neovascular age-related macular degeneration has now been confirmed in a clinical trial demonstrating that multiple intravitreal injections of pegaptanib, an aptamer that binds VEGF, slows the rate of visual loss (Gragnoudas et al., 2004). This is a useful start to pharmacotherapy for choroidal neovascularization, but further improvements are needed. Regression of choroidal neovascularization is not achieved in patients treated with pegaptanib; the size of lesions continued to increase over the course of a year, although at a slower rate than that seen in patients that did not receive pegaptanib. Antagonism of VEGF seems to reduce excessive permeability and slows growth, but does not cause involution of choroidal neovascularization. VEGF provides some survival signals to newly developed endothelial cells (Alon et al., 1995), but survival signals from the extra-cellular matrix may be sufficient to allow continued growth of choroidal neovascular lesions, although at a slower rate. Combination treatment using JSM6427 with a VEGF antagonist may be extremely useful because perturbation of at least one source of matrix-derived survival signaling along with elimination of the survival signal provided by VEGF may be an effective means to promote involution of choroidal neovascularization.

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References


αβ1 Integrin and Ocular Neovascularization

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