SB-267268, a Nonpeptidic Antagonist of \( \alpha_\nu \beta_3 \) and \( \alpha_\nu \beta_5 \) Integrins, Reduces Angiogenesis and VEGF Expression in a Mouse Model of Retinopathy of Prematurity

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Purpose. To determine whether SB-267268, a nonpeptidic antagonist of the \( \alpha_\nu \beta_3 \) and \( \alpha_\nu \beta_5 \) integrins, attenuates angiogenesis in a murine model of retinopathy of prematurity (ROP) and alters the expression of vascular endothelial growth factor (VEGF) and its second receptor (VEGFR-2).

Methods. In receptor binding, SB-267268 exhibited nanomolar potency for human, monkey, and murine \( \alpha_\nu \beta_3 \) and \( \alpha_\nu \beta_5 \). SB-267268 inhibited the attachment of \( \alpha_\nu \beta_3 \)-transfected HEK293 cells to microtiter plate wells precoated with RGD-containing matrix proteins, and vitronectin-mediated human and rat aortic smooth-muscle-cell migration. At postnatal day (P)12, C57BL/6 mice were exposed to 80% oxygen for 7 days followed by 7 days in room air (angiogenic period). Between P12 and P17, ROP mice were administered sterile saline (vehicle intraperitoneal [i.p.]) or SB-267268 (60 mg/kg bi-daily, i.p.). Shams were exposed to room air from P0 and administered either vehicle or SB-267268 during P12 to 17. In at least 3 randomly chosen paraffin sections from each eye, the number of blood vessel profiles in the inner retina were counted. In situ hybridization for VEGF and VEGFR-2 was performed on at least 8 randomly chosen paraffin sections from each eye.

Results. SB-267268 reduced pathologic angiogenesis in ROP mice by approximately 50% and had no effect on development of retinal angiogenesis in shams. Both VEGF and VEGFR-2 mRNA were upregulated in the inner retina of ROP mice and reduced with SB-267268.

Conclusions. Nonpeptidic inhibition of \( \alpha_\nu \beta_3 \) and \( \alpha_\nu \beta_5 \) integrins is effective in ROP and may be a suitable anti-angiogenic therapy for other ischemic retinal pathologies. (Invest Ophthalmol Vis Sci. 2006;47:1600–1605) DOI:10.1167/iovs.05-1314

Integrins are a family of multifunctional cell-adhesion molecules composed of noncovalently associated \( \alpha \) and \( \beta \) chains. They are transmembrane receptors that bind extracellular matrix components, including vitronectin, fibronectin, laminin, collagen, fibrinogen, and thrombospondin. Integrins are involved in the regulation of a wide variety of cellular events, including adhesion, migration, invasion, proliferation, and cell survival and apoptosis. A number of studies have implicated the \( \alpha_\nu \beta_3 \) and \( \alpha_\nu \beta_5 \) integrins in angiogenesis. \( \alpha_\nu \beta_3 \) is located on actively proliferating endothelial cells in human diabetic retinopathy and retinopathy of prematurity (ROP), and in human wound granulation tissue and chick chorioallantoic membrane. Inhibition of \( \alpha_\nu \) integrins with cyclic peptide antagonists and antibodies reduces angiogenesis in solid tumors and is associated with downregulation of the potent angiogenic and permeability factor, vascular endothelial growth factor (VEGF). These findings have suggested that inhibition of \( \alpha_\nu \) integrins may be an appropriate strategy for organ protection in a variety of angiogenic pathologies.

Angiogenesis is the hallmark feature of ischemic retinopathies such as ROP and proliferative diabetic retinopathy. In both diseases, new blood vessels proliferate in the inner retina and penetrate the vitreous cavity. These blood vessels are abnormally formed, leading to sight-threatening hemorrhage and edema. In these instances, pathologic angiogenesis is linked with upregulation of VEGF and its second receptor, VEGFR-2. \( \alpha_\nu \beta_3 \) and \( \alpha_\nu \beta_5 \) integrins may participate in the pathologic angiogenesis that occurs in ROP and proliferative diabetic retinopathy. For example, in mouse models of ROP, the administration of cyclic peptide antagonists of \( \alpha_\nu \) integrins and \( \alpha_\nu \beta_3 \) attenuates retinal angiogenesis and also reduces normal developmental vascularization of the retina in mice.

SB-267268 is a small molecule antagonist of both \( \alpha_\nu \beta_3 \) and \( \alpha_\nu \beta_5 \) integrins whose structure is based on a 2-benzazepine template (Fig. 1). The aim of the present study was to determine whether administration of SB-267268 reduces retinal angiogenesis in mice with ROP and alters retinal VEGF and VEGFR-2 expression.

Methods

Animals

Pregnant female C57BL/6 mice were obtained from the Animal Resource Centre, Perth, Western Australia. The mothers were randomly divided into 4 experimental groups with 7–9 pups per group. These groups consisted of (1) sham treated with vehicle (n = 9), (2) sham treated with the \( \alpha_\nu \beta_3 \beta_5 \) integrin antagonist SB-267268 (n = 7); (3) ROP treated with vehicle (n = 8), and (4) ROP treated with SB-267268 (n = 9). Shams were mice exposed to room air from birth until P17. The ROP model in mice followed a previously published method. Seven-day-old pups and their mother were housed in sealed chambers that contained \( 75 \pm 5\% \) \( O_2 \) and 2% \( CO_2 \), using medical grade \( O_2 \) and industrial grade air. Gas levels in the chamber were monitored twice daily by using a gas analyzer (ML 205; AD Instruments, Pty. Ltd., Australia) and a chart recorder (Chart v3.5 program on the MacLab/2E System; AD Instruments, Bella Vista, NSW, Australia). An airflow rate of
were consistent with the guidelines set by the Australian National chamber and placed in room air for 2 hours. Experimental procedures standard mice chow (GR2; Clark-King and Co., Gladesville, Victoria, from human placenta, and a IIbb3 purified from human platelets. After [3H]-SKF107260 was solubilized and counted. The binding of were aspirated completely, washed twice with Buffer A, and bound by the addition of [3H]-SKF107260 (5 nM). After incubation, the wells concentrations of the compound were added to the wells and followed 96-well plates were coated with integrins αvb3, αvb5, and α5β1 purified (in the case of 6xhis-tagged mouse/H9251

were fixed by the addition of 25 μL of a 10% formaldehyde solution, pH 7.4, at room temperature for 10 minutes. The plates were washed three times with 0.2 mL of PBS with 0.1% BSA, and the adherent cells were stained with 0.1 mL of 0.5% toluidine blue for 20 minutes at room temperature. Excess stain was removed by extensive washing with deionized water, and toluidine blue incorporated into cells was eluted by the addition of 0.1 mL of 50% ethanol that contained 50 mM HCl and was quantitated by measuring absorbance at 630 nm on a microtiter plate reader (TiterTek Multiskan MC, Sterling, VA).

Cell Migration Assay
Human and rat aortic smooth-muscle–cell (SMC) migration was evaluated as described previously. Briefly, migration of SMCs was examined in Transwell culture chambers by using a polycarbonate membrane with pores of 8 microns (Costar; Corning, Cambridge, MA). The SMCs were suspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.2% BSA at a concentration of 2.5 × 10⁶ cells/mL. In the standard assay, 0.2 mL human or rat SMC was placed in the upper compartment of the chamber. The lower compartment contained 0.6 mL DMEM supplemented with 0.2% BSA, vitronectin, and SB-267268 or vehicle. The chambers were incubated at 37°C in an atmosphere of 95% air and 5% CO₂. After incubation, nonmigrated cells on the upper surface of the membrane were scraped gently and washed with PBS. The filters were then fixed with methanol and stained with Giemsa. The number of SMCs per ×150 high-power field that had migrated to the lower surface of the filters was determined microscopically. Four randomly chosen high-powered fields were counted per filter.

Histopathology
After the 17-day experimental period, the mice were killed by an i.p. injection of 120 mg/kg per body weight pentobarbital sodium (Nembutal; Rhone Merieux, Queensland, Australia). Both eyes from each animal were removed and fixed for 3 hours in 4% paraformaldehyde fixative in 0.1M phosphate buffer. The eyes were processed in graded alcohols before being embedded in paraffin wax. The eyes were then serially sectioned at 3 μm, 90° to the optic nerve and placed on 3 aminopropyl-triethoxysilane (Sigma, St. Louis, MO) coated slides. Approximately 120 sections/eye were collected and incubated overnight at 37°C.

Three sections from 1 eye from each animal were randomly chosen, deparaffinized, and stained with Mayer’s hematoxylin (5 minutes) and eosin (5 minutes) (Amber Scientific Laboratories, Belmont, Australia), and coverslipped. By using an established technique, blood vessel profiles (BVP) were counted in the inner retina and included vessels adherent to the inner limiting membrane (ILM). The inner retina comprised the ILM, the ganglion cell layer (GCL), the inner plexiform layer (IPL), and the inner nuclear layer (INL). Four nonoverlapping fields per section were evaluated in a masked manner. A BVP was defined as an endothelial cell (stained blue) or a blood vessel with a lumen. Counting was performed on a photomicroscope (Olympus BH-2; Olympus, Tokyo, Japan) at a magnification of ×40, and images were captured on a digital camera connected to an IBM computer (Spot digital camera; SciTECH Pty. Ltd., Preston, Victoria, Australia). Two investigators masked to the experimental groups counted BVPs.

Receptor Binding Assays
The affinity of SB-267268 for various integrins was determined by using binding assays, as described previously, using purified receptors and a RGD-containing cyclic peptide [3H]-SKF107260 as the ligand. Briefly, 96-well plates were coated with integrins αβ3, αβ5, and αβ1 purified from human placenta, and αβ3β1 purified from human plateletlets. After blocking of nonspecific sites with Buffer A (50 mM Tris-HCl, 100 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂, pH 7.4) containing 3% BSA, various concentrations of the compound were added to the wells and followed by the addition of [3H]-SKF107260 (5 nM). After incubation, the wells were aspirated completely, washed twice with Buffer A, and bound [3H]-SKF107260 was solubilized and counted. The binding of SB-267268 to mouse integrins was assessed in an ELISA-type integrin/ligand binding assay by using a commercial platform, MesoScale Discovery (MSD), Gaithersburg, MD. The assay used either native integrin protein or recombinant purified integrin protein. Recombinant protein was derived from a baculovirus expression system and comprised the extracellular domain fused to a fos/jun dimerization cassette and His/FLAG tags. Recombinant fibronectin protein expressed in Escherichia coli as a GST fusion protein (GST-fibronectin) was used as a ligand. In the assay, 5 ng/well integrin protein was bound directly to the electrode (in the case of human αβ3 and αβ5 proteins) or was added to wells of MSD plates coated with 10 ng/well anti-6xhis capture antibody (in the case of 6xhis-tagged mouse αβ3, mouse αβ3, and rat αβ3 proteins). SB-267268, 10 nM biotinylated fibronectin, and 20 nM ruthenylated streptavidin were added, and the plates were incubated for 4 hours. Read buffer was then added, and the plates were read on the MSD instrument. SB-267268 inhibition of integrin/ligand binding was measured as a decrease in the signal in the assay. Data were analyzed with a commercial software (ActivityBase; NovaScreen, Hanover, MD), using a 4-parameter logistic equation for curve fitting.

Cell Adhesion Assays
The effect of SB-267268 on the adhesion of cells was determined in a cell adhesion assay by using HEK-293 cells cotransfected with human αα and ββ, Briefly, 96-well plates were coated overnight with 0.2 μg/mL of human vitronectin or rat osteopontin. The plates were washed once with PBS and blocked with 5% BSA, followed by the addition of ααββ, expressing HEK293 cells (50,000 cells) in Roswell Park Memorial Institute (RPMI); 20 mM HEPES, pH 7.4, and 0.1% BSA were added to the well. After 1 hour of incubation at 37°C, the cells were fixed by the addition of 25 μL of a 10% formaldehyde solution, pH 7.4, at room temperature for 10 minutes. The plates were washed three times with 0.2 mL of PBS with 0.1% BSA, and the adherent cells were stained with 0.1 mL of 0.5% toluidine blue for 20 minutes at room temperature. Excess stain was removed by extensive washing with deionized water, and toluidine blue incorporated into cells was eluted by the addition of 0.1 mL of 50% ethanol that contained 50 mM HCl and was quantitated by measuring absorbance at 630 nm on a microtiter plate reader (TiterTek Multiskan MC, Sterling, VA).

SB-267268 was provided by GlaxoSmithKline (CVU CEDD; King of Prussia, PA). SB-267268 was dissolved in sterile water and administered at a dose of 60 mg/kg bi-daily to pups by intraperitoneal (i.p.) injection. Sham pups were administered sterile water (vehicle) bi-daily by i.p. injection. SB-267268 and vehicle were administered at 0830 hours and 0430 hours each day. The injection volume was 100 μL.

During the experiment, mothers were provided with water and standard mice chow (GR2; Clark-King and Co., Gladesville, Victoria, Australia) ad libitum and were exposed to normal 12-hour light/dark cycles. Pups received nutrition from their mothers. To avoid respiratory distress, each day, mother and pups were removed from the chamber and placed in room air for 2 hours. Experimental procedures were consistent with the guidelines set by the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Receptor Binding Assays
The affinity of SB-267268 for various integrins was determined by using binding assays, as described previously, using purified receptors and a RGD-containing cyclic peptide [3H]-SKF107260 as the ligand. Briefly, 96-well plates were coated with integrins αβ3, αβ5, and αβ1 purified from human placenta, and αβ3β1 purified from human plateletlets. After blocking of nonspecific sites with Buffer A (50 mM Tris-HCl, 100 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂, pH 7.4) containing 3% BSA, various concentrations of the compound were added to the wells and followed by the addition of [3H]-SKF107260 (5 nM). After incubation, the wells were aspirated completely, washed twice with Buffer A, and bound [3H]-SKF107260 was solubilized and counted. The binding of SB-267268 to mouse integrins was assessed in an ELISA-type integrin/ligand binding assay by using a commercial platform, MesoScale Discovery (MSD), Gaithersburg, MD. The assay used either native integrin protein or recombinant purified integrin protein. Recombinant protein was derived from a baculovirus expression system and comprised the extracellular domain fused to a fos/jun dimerization cassette and His/FLAG tags. Recombinant fibronectin protein expressed in Escherichia coli as a GST fusion protein (GST-fibronectin) was used as a ligand. In the assay, 5 ng/well integrin protein was bound directly to the electrode (in the case of human αβ3 and αβ5 proteins) or was added to wells of MSD plates coated with 10 ng/well anti-6xhis capture antibody (in the case of 6xhis-tagged mouse αβ3, mouse αβ3, and rat αβ3 proteins). SB-267268, 10 nM biotinylated fibronectin, and 20 nM ruthenylated streptavidin were added, and the plates were incubated for 4 hours. Read buffer was then added, and the plates were read on the MSD instrument. SB-267268 inhibition of integrin/ligand binding was measured as a decrease in the signal in the assay. Data were analyzed with a commercial software (ActivityBase; NovaScreen, Hanover, MD), using a 4-parameter logistic equation for curve fitting.
In Situ Hybridization for VEGF and VEGFR-2

Riboprobes were synthesized from cDNAs encoding mouse VEGF and VEGFR-2 (a gift from Steven Stacker, Ludwig Institute, Parkville, Australia). The cDNAs were cloned into pGEM Z (Promega, Madison, WI) and linearized with HindIII to produce antisense probes by using a polymerase (SP6 RNA polymerase; Promega). Three-μm paraffin sections of eye premounted on 1% 3-aminopropyltriethoxysilane coated slides were dewaxed, rehydrated in graded ethanol and milliQ water, equilibrated in P buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA), and incubated in 125 μg/mL Pronase E (Bio Scientific, Gynea, NSW, Australia) in P buffer for 10 minutes at 37°C. Sections were then washed in 0.1 M sodium phosphate buffer (pH 7.2), briefly refixed in 4% paraformaldehyde (Crown Scientific Pty. Ltd, Scoresby, Victoria, Australia) for 10 minutes, rinsed in milliQ water, dehydrated in 70% ethanol, and air-dried. Hybridization buffer containing 2 × 10⁴ cpm/μL riboprobe in 300 mM NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM Na₂HPO₄, 5 mM EDTA (pH 8.0), 1 × Denhardt’s solution, 50% formamide, 17 mg/mL yeast RNA, and 10% wt/vol dextran sulfate was heated to 85°C for 5 minutes, and 25 μL of this solution was then added to each section. Hybridization was performed overnight at 60°C in 50% formamide-humidified chambers. Sections hybridized with sense probes for VEGF and VEGFR-2 were used as controls for non-specific binding. After hybridization, slides were washed in ×2 SSC containing 50% formamide prewarmed to 50°C to remove the cover-slips. Sections were then washed in the above-described solution for 1 hour at 55°C, rinsed three more times in RNase buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 0.5 M NaCl), and incubated with RNase A (150 μg/mL) for 1 hour at 37°C. Sections were later washed in ×2 SSC for 45 minutes at 55°C, dehydrated in graded ethanol, air-dried, and exposed to autoradiographic film (Kodak X-Omat; Kodak, Rochester, NY) for 5 days. Slides were subsequently dipped in emulsion (Ilford LM1; Ilford, Cheshire, UK), stored in a light-free box with desiccant at 4°C for 4 weeks, immersed in developer (Kodak D19; Kodak, Rochester, NY), fixed (Ilford Hypam; Ilford), and stained with hematoxylin and cosin.

Dark field images were captured by using light microscopy and a digital camera (Fujix HC-2000; Fuji, Tokyo, Japan). The outline of the inner retina (ILM, GCL, IPL, and INL) was defined by interactive tracing. Gene expression was then quantitatively measured to determine the proportion of the area occupied by autoradiographic grains as previously described by using computerized image analysis (AIS: Imaging Research, Ontario, Canada). All sections were hybridized to their respective probes in the same experiment and analyzed in duplicate (n = 8 sections per rat, 6 to 8 rats per group). All analyses were done with the observer masked to the animal study group.

Statistics

All values are expressed as mean ± SEM. Data were analyzed by ANOVA followed by a Fisher’s post hoc comparison, with a value of P < 0.05 considered statistically significant. A statistics program (Statview for Windows, Version 5.0.1; SAS Institute Inc., Cary, NC) was used to analyze data.

Results

Receptor Binding, Cell Adhesion, and Cell Migration

In a radioligand displacement assay, SB-267268 potently displaced [³H]-SKF107260 binding to human and monkey α₃β₃ and human α₅β₃ with Ki values of 0.9 nM, 0.5 Nm, and 0.7 nM, respectively. However, evaluation of SB-267268 on [³H]-SKF107260 binding to related integrin receptors demonstrated >1000-fold selectivity for the human α₃β₃ receptor versus the human α₅β₃, α₅β₇, and α₅β₁ receptors (Table 1). In further binding assays with native or recombinant nonlabeled integrins and fibronectin as ligand, SB-267268 inhibited the binding of fibronectin to human and mouse α₅β₃ with IC₅₀ values of 0.68 nM and 0.29 nM, respectively. SB-267268 was much less potent for inhibition of human, mouse, and rat α₅β₃ integrin (see Table 1). Consistent with the binding data, using an in vitro cell adhesion assay format, SB-267268 inhibited the attachment of both α₅β₃-transfected HEK293 cells to microroller plate wells precoated with arginine-glycine-aspartic acid (RGD)-containing matrix proteins with IC₅₀ values of 12 nM (see Table 1). SB-267268 also inhibited vitronectin-mediated human and rat aortic SMC migration with IC₅₀ values of approximately 12.3 nM and 3.6 nM, respectively. The effect of SB-267268 on α₅β₃-mediated cell adhesion and migration appeared to be relatively specific, because this compound did not exhibit any appreciable activity when profiled in a screen against a panel of 46 receptors and 9 enzymes (data not shown).

Body Weight

At P17, there was no significant difference in body weight between the groups of animals; sham + vehicle (7.15 ± 0.11 g), sham + SB-267268 (7.13 ± 0.15 g), ROP + vehicle (7.05 ± 0.08 g), and ROP + SB-267268 (7.10 ± 0.10 g).

BVPs in the Inner Retina

The results are shown in Figure 2. In sham mice treated with vehicle, BVPs were observed in the inner retina. SB-267268 treatment to sham mice did not change the appearance of the retina or the density of BVPs in the inner retina. In ROP mice treated with vehicle, the number of BVPs increased in the retina or the density of BVPs in the inner retina. In ROP mice treated with SB-267268, BVPs in the inner retina by 50% compared with ROP vehicle.

VEGF and VEGFR-2 Gene Expression

The results are shown in Figures 3 and 4. Intense VEGF and VEGFR-2 expression was detected in the INL of all retinas from all groups, with less expression observed in the GCL. In sham mice, SB-267268 did not reduce VEGF or VEGFR-2 gene expression in the inner retina. In ROP mouse treated with vehicle, both VEGF and VEGFR-2 mRNA increased in the INL and GCL compared with all sham groups. In ROP mice treated with SB-267268, VEGF and VEGFR-2 gene expression in the INL and GCL was reduced, with VEGFR-2 mRNA levels similar to sham groups.

Table 1. SB-267268 Binding to Human, Monkey and Mouse Integrins

<table>
<thead>
<tr>
<th>Receptor Binding Studies</th>
<th>Activity (Ki, nM)†</th>
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<tr>
<td>Human α₃β₃ binding</td>
<td>0.9</td>
</tr>
<tr>
<td>Monkey α₃β₃ binding</td>
<td>0.5</td>
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<tr>
<td>Human α₅β₃ binding</td>
<td>0.7</td>
</tr>
<tr>
<td>Human α₅β₇ binding</td>
<td>1000</td>
</tr>
<tr>
<td>Human α₅β₁ binding</td>
<td>2000</td>
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<tr>
<td>Human α₅β₅ binding</td>
<td>&gt;1000</td>
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‡ Data represent the IC₅₀ of SB-267268 (nM) required for inhibition of integrin binding to various integrins.

* Data represent displacement of [³H]-SKF107260 binding to integrin receptors by SB-267268.
Vessel formation and maturation in a variety of tissues. 5,7,12,19 are potential targets, because they are implicated in new blood compared with sham groups.

Because of its selective activity on VEGFR-2 system. The effect of SB-267268 in ROP appears to be SB-267268 in ROP may involve downregulation of the VEGF/angiogenesis in neonatal mice. The anti-angiogenic effects of nist, SB-267268, reduces pathologic but not developmental administration of a nonpeptidic combined therapies that prevent the adhesion of vascular endothelial
treatment of ischemic retinopathies, for example, proliferative diabetic retinopathy.

Evidence that α, β integrins are important in retinal angiogenesis is provided by studies in mouse models of ROP, where ligation inhibition of α, type integrins with cyclic penta-petid peptide reduces angiogenesis when administrated by either i.p. injection or topically. Other studies have highlighted the importance of α, β integrins in ischemic retinopathies. The α, β integrins have been localized to neovascular tissue removed from the retinal surface during vitrectomy from patients with proliferative diabetic retinopathy and in new vessels in mice with ROP. Similarly, α, β integrin and its major ligand, vitronectin, as well as α, β integrins are found in both basal and luminal surfaces of endothelial cells in vascularized tissues from patients with proliferative diabetic retinopathy. The efficacy of inhibition of α, β integrins has been evaluated panel of approximately 50 other receptors and enzymes. Overall, these findings have relevance for the development of non-invasive therapies for ischemic retinopathies, for example, proliferative diabetic retinopathy.

DISCUSSION

Anti-angiogenic therapies are currently being evaluated for the treatment of ischemic retinopathies, for example, proliferative diabetic retinopathy. One approach is the development of therapies that prevent the adhesion of vascular endothelial cells to the extracellular matrix. The α, β and α, β integrins are potential targets, because they are implicated in new blood vessel formation and maturation in a variety of tissues. 5,7,12,19 The major results from the present study are that chronic administration of a nonpeptidic combined α, β/α, β antagonist, SB-267268, reduces pathologic but not developmental angiogenesis in neonatal mice. The anti-angiogenic effects of SB-267268 in ROP may involve downregulation of the VEGF/VEGFR-2 system. The effect of SB-267268 in ROP appears to be because of its selective activity on α, β and α, β integrins, because, in vitro, this compound demonstrated potent and selective binding to α, β integrins, and α, β receptors and inhibition of cell adhesion and migration. SB-267268 exhibits greater than 1000-fold selectivity for other integrins and no activity on a

FIGURE 2. Three micron paraffin sections of retina from mice with ROP and treated with SB-267268. Quantitation of BVPs in the inner retina. Magnification, x125. Scale bar, 50 μm. Sections stained with hematoxylin and cosin. (A) Sham + vehicle. (B) Sham + SB-267268. (C) ROP + vehicle. (D) ROP + SB-267268. Single arrows denote blood vessels in the inner retina. Double arrows denote blood vessels extending from the retina into the vitreous cavity. In ROP + vehicle (B), numerous blood vessels are found in the inner retina and penetrating into the vitreous cavity compared with all other groups. Values are means ± SEM; n = 7 to 9 animals per group. *P < 0.005 compared with sham groups. #P < 0.001 compared with SB-267268. †P < 0.0001 compared with SB-267268.

FIGURE 3. Dark field micrographs of retina showing VEGF gene expression in paraffin sections from neonatal mice with ROP and treated with SB-267268. Retinal pigment epithelium. Magnification, x125. Scale bar, 50 μm. (A) Sham + vehicle. (B) Sham + SB-267268. (C) ROP + vehicle. (D) ROP + SB-267268. VEGF mRNA is similar in (A) and (B), with intense expression in the INL and less mRNA in the GCL. VEGF mRNA is increased with ROP (C) and reduced in ROP mice treated with SB-267268 (D). Values are means ± SEM; n = 7 to 9 animals per group. dpm/mm², disintegration per minute of 125 I per mm² of tissue. *P < 0.005 compared with sham groups. #P < 0.001 compared with SB-267268. †P < 0.001 compared with untreated ROP.
the Bcl:Bax ratio, with a consequent anti-apoptotic effect.26 In angiogenesis and tumor regression 7 and improved arthritic more recent years, a humanized monoclonal IgG1 antibody that reduces artery size in balloon-injured hypercholesterolemic rats with laser-induced choroidal angiogenesis, a cyclic antagonist inhibits the progression of the lesion. 24 These find-

when administered by either i.p. or periocular injection.4 In XJ735 given to mice with ROP reduces retinal angiogenesis and in animal models of retinal angiogenesis. A cyclic antagonist XF735 given to mice with ROP reduces retinal angiogenesis when administered by either i.p. or periocular injection.5 In rats with laser-induced choroidal angiogenesis, a cyclic α5β3 antagonist inhibits the progression of the lesion.24 These findings are consistent with studies in other experimental models in which α5β3 antibodies, for example, LM609, decreased angiogenesis and tumor regression7 and improved arthritic disease.25 The anti-angiogenic properties of α5β3 is most likely to be caused by the suppression of the activity of p53 and p53-inducible cell-cyclic inhibitor p21WAF/CIP and increases in the Bcl: Bax ratio, with a consequent anti-apoptotic effect.26 In more recent years, a humanized monoclonal IgG1 antibody that binds human integrin α5β3 has been developed. In preclinical studies, a monoclonal antibody (Vitaxin, Gaithersburg, MD) reduces artery size in balloon-injured hypercholesterolemic rabbits,27 which may be caused by a decrease in transforming growth factor β1 and an increase in cellular apoptosis. Vitaxin has also been used in a Phase I clinical trial of patients with late stage cancer. Vitaxin was nontoxic and potentially active in patients with progressive tumors with stage IV disease.28

Like α5β3 integrins, αβ3 integrins are implicated in angiogenesis,19,20 α5β3 integrins have been identified as the principal α3 integrin associated with endothelial cells in the corneal alkaline burn model of inflammation-mediated angiogenesis.29 In the retina, α5β3 integrins have been localized to blood vessels and nonvascular areas in proliferative diabetic retinopathy.12 To date, few studies have examined the effects of combined α5β3 and αβ3 antagonism on retinal angiogenesis. In rodents, the retinal vasculature develops after birth and is complete by 2 weeks of age.20 In a study of developmental retinal vasculogenesis in mice, cyclic peptide antagonists to α5β3 and αβ3 administered from P0 to P4, reduced normal retinal vascularization. In the present study, SB-267268 did not alter developmental retinal vasculogenesis in sham animals when administered between P11 to P17. The reasons for the discrepancies between the 2 studies are not clear but may be because of the timing of delivery of the combined α5β3 and αβ3 integrin inhibitors. In rodents, the majority of retinal vasculogenesis occurs within the first week to 10 days of the postnatal period,30 and, therefore, it is during this period that new blood vessels may be most responsive to combined α5β3 and αβ3 integrin antagonism. Of interest is that both the cyclic peptide antagonist to α5β3 and αβ320 and SB-267268 reduced to a similar extent pathologic angiogenesis in murine ROP. In these studies, the integrin antagonists were administered by either subcutaneous or i.p. injection.20,21 SB-267268 has a high affinity for α5β3 and αβ3 integrins and good pharmokinetics, which include moderate plasma clearance and high oral bioavailability.13 These features suggest that SB-267268 may have more potential as a treatment for ischemic retinopathies than existing integrin antagonists. Both VEGF and basic fibroblast growth factor (bFGF) are involved in developmental and pathologic angiogenesis.31–35 In mice with ROP, temporal changes in these growth factors occurs after the removal of pups from a hyperoxic environment to room air.21 Retinal VEGF expression increases immediately after the return of mice to room air, whereas bFGF is predominately expressed later when retinal angiogenesis is maximal.21 In earlier studies, 2 growth-factor-dependent angiogenic pathways for α5β3 and αβ3 were described.19 α5β3 integrins have been reported to mediate bFGF or TNF-α induced angiogenesis, whereas αβ3 mediated angiogenesis involved VEGF and TGF-α. Subsequent studies have reported that in ROP, administration of inhibitors of α3-type integrins to mice soon after the return of pups to room air was associated with a 57% reduction in new vessels, whereas a late intervention had no effect.23 These findings were interpreted as indicating that α3-integrin inhibition is largely VEGF dependent. The results of the present study are consistent with these findings. As previously described in studies by ourselves11,18 and other investigators,34,35 ROP was associated with an increase in VEGF and VEGFR-2 expression in the inner retina. In the present study, SB-267268 reduced VEGF and VEGFR-2 expression in ROP. In support of these findings, there is evidence that platelet-derived growth factor receptor α and VEGFR-2 associate with the extra-

celluar domain of the β3 integrin subunit.36 In addition, Soldi et al.37 have shown that anti-α5β3 antibodies can inhibit VEGF-induced VEGFR-2 phosphorylation and cell migration when endothelial cells are bound to the α5β3 ligand vitronectin.

In summary, the findings of the present study indicate that SB-267268 is effective in reducing pathologic angiogenesis in a model of ischemic retinopathy and that its beneficial effects most likely involve inhibition of retinal VEGF and VEGFR-2. SB-267268 may be a useful treatment for ischemic retinopathies, for example, proliferative diabetic retinopathy because of its high oral bioavailability13 compared with other integrin
antagonists that are administered by either a systemic or intracocular route.

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