Integrin α2β1 Is the Required Receptor for Endorepellin Angiostatic Activity*

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Endorepellin, the C-terminal module of perlecans, has angiostatic activity. Here we provide definitive genetic and biochemical evidence that the functional endorepellin receptor is the α2β1 integrin. Notably, the specific endorepellin binding to the receptor was cation-independent and was mediated by the α2 I domain. We show that the anti-angiogenic effects of endorepellin cannot occur in the absence of α2β1. Microvascular endothelial cells from α2β1−/− mice, but not those isolated from either wild-type or α1β1−/− mice, did not respond to endorepellin. Moreover, syngeneic Lewis lung carcinoma xenografts in α2β1−/− mice failed to respond to systemic delivery of endorepellin. In contrast, endorepellin inhibited tumor growth and angiogenesis in the wild-type mice expressing integrin α2β1. We conclude that the angiostatic effects of endorepellin in vivo are mediated by a specific interaction of endorepellin with the α2β1 integrin receptor.

The incorporation of new blood vessels into growing neoplasms is a prerequisite for tumor viability and progression. Accordingly, much attention has been invested in the search for and characterization of anti-angiogenic agents to enable regulated and inhibited tumor angiogenesis as part of cancer therapies (1). The proteoglycan perlecans plays a key role in the angiogenic process, primarily by modulating the availability and activity of growth factors involved in angiogenesis such as fibroblast growth factor 2, VEGF,3 and platelet-derived growth factor (2–6). The most C-terminal part of perlecans (domain V), named endorepellin, is a powerful angiogenic inhibitor (7). Endorepellin carries three laminin-like globular (LG) domains separated by epidermal growth factor-like repeats (8) and binds to numerous extracellular matrix proteins, growth factors, and receptors including collagen XVIII, fibulin-2, nidogen, fibroblast growth factor 7, fibroblast growth factor-binding protein, ECM1 (7, 9–12), α-dystroglycan, and integrin α2β1 (9, 13–16). The endorepellin anti-angiogenic effect is parallel to several proteolytically released fragments from vascular basement membrane such as endostatin, the NC-1 domain of collagen type XVIII, and tumstatin, the NC-1 domain of type IV collagen α3 chain (8, 17, 18). These fragments principally act on endothelial cells as “negative” ligands for specific integrin receptors. Endorepellin is a potent inhibitor in several angiogenesis assays such as endothelial cell migration, collagen-induced capillary morphogenesis, blood vessel recruitment into Matrigel plugs, and chicken chorioallantoic membrane (7, 19). It also effectively retards in vivo tumor growth by specifically targeting tumor angiogenesis (20). We hypothesize that endorepellin takes effect via the LG3 domain binding to the integrin α2β1 causing actin disassembly and therefore affecting three key steps of angiogenesis: endothelial cell adhesion, migration, and morphogenesis.

Here we have further investigated the endorepellin–α2β1 integrin interactions by using cell-free experiments with a soluble form of the α2β1 integrin, by in vitro assays of endothelial cells deficient in the integrin α1 or α2 subunits, by siRNA knockdown of the integrin α2 subunit, and in syngeneic tumor xenografts growing in mice lacking the α2β1 integrin receptor. We report a novel cation-independent binding between endorepellin and integrin α2 I domain and show that integrin α2β1 is necessary for recruitment of endorepellin to the vasculature and for its anti-angiogenic properties both in vitro and in vivo. The presented studies increase both the knowledge about this specific angiogenesis inhibitor and angiogenesis in general.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**—HUVEC at passages 1–6, HT1080, and LLC cells were cultured under standard conditions. Human recombinant endorepellin and LG3 harboring a His6 tag at their C termini were purified on a nickel-nitrilotriacetic acid resin column as previously described (7). The soluble ectodomain heterodimer of the α2β1 integrin was generated as previously described (21). The recombinant production of the...
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oligo-His-tagged integrin \( \alpha 2 \) I domain was carried out similar to the glutathione S-transferase-tagged \( \alpha 2 \) I domain (22). The binding activity of both integrin constructs was tested on collagen I and rhodocetin, a high affinity \( \alpha 2 \beta 1 \) integrin-specific antagonist (21).

**Binding Assays with Recombinant \( \alpha 2 \beta 1 \) Integrin and \( \alpha 2 \) I Domain—**A binding assay of soluble \( \alpha 2 \beta 1 \) integrin to immobilized collagen I or endorepellin in the presence of either 1 \( \mu \)M MnCl\(_2\), 1 \( \mu \)M MgCl\(_2\), and integrin-activating antibody 9E9G7 or 10 \( \mu \)M EDTA was performed as previously published (21). For the reciprocal binding assays, recombinant \( \alpha 2 \beta 1 \) integrin ectodomain heterodimer or oligo-His-tagged integrin \( \alpha 2 \) I domain was coated onto a microtiter plate at 5 \( \mu \)g ml\(^{-1}\) in Tris-buffered saline/magnesium/manganese buffer (2 \( \mu \)M MgCl\(_2\), 1 \( \mu \)M MnCl\(_2\), pH 7.4) at 4°C overnight. After blocking with 1% BSA in Tris-buffered saline/magnesium/manganese buffer, the wells were incubated with endorepellin at the indicated concentrations or with 10 \( \mu \)g ml\(^{-1}\) CB3[IV], both in blocking buffer. This soluble collagen IV fragment harbors the \( \alpha 2 \beta 1 \) integrin-binding site (23, 24) and was used as positive control. After washing, bound integrin ligands were fixed with 2.5% glutaraldehyde in HEPES-buffered saline (50 mM HEPES/HCl, 150 mM NaCl, 2 mM MgCl\(_2\), 1 mM MnCl\(_2\), pH 7.4) for 10 min and quantified by enzyme-linked immunosorbent assay using polyclonal rabbit antibodies against endorepellin and CB3[IV] as primary antibodies and secondary alkaline phosphatase-coupled antibodies directed against rabbit immunoglobulins. The plates were read at 405 nm.

**Cell Adhesion and Actin Disassembly Assays—**CC2-treated chamber slides (Nunc Inc., Roskilde, Denmark) were coated with 100 \( \mu \)g ml\(^{-1}\) rat tail collagen I (BD Biosciences, Bedford, MA) at 4°C overnight. The wells were washed with PBS, and 10\(^5\) HUVEC were seeded. After 24 h, the medium was changed to serum-free M199 for 30 min prior to treatment with 150 \( \mu \)M endorepellin, 150 \( \mu \)M endorepellin, and 1 \( \mu \)M human recombinant integrin \( \alpha 2 \) I domain or 1 \( \mu \)M human recombinant integrin \( \alpha 2 \) I domain alone for 30 min at 37°C. The medium was removed, and M199 containing the prior incubated reagents were added for 25 min at 37°C. The nuclei and actin stress fibers were visualized by DAPI and fluorescein isothiocyanate-phalloidin staining, respectively (14). For quantification of cell adhesion, the cells were counted by their DAPI staining. For quantification of actin stress fibers, 80–100 randomly selected cells of each group were analyzed. The assays were performed in triplicate.

**siRNA Transfection of HUVEC and HT1080 Cells—**HUVEC/HT1080 were plated at subconfluent density on 12-well culture plates (Nunc). The cells were allowed to grow in complete medium until ∼70% confluence. Following washing, the cells were transfected with two validated siRNA constructs (Ambion Applied Biosystems, Foster City, CA), targeting different regions of the integrin \( \alpha 2 \) mRNA. siRNA\(^{1}\) (antisense 5’-3’, UCUGAAAGUUGUCUCUCCTG) and siRNA\(^{2}\) (antisense 5’-3’, AACACUUCUCCUGUUGUACCT) target exons 10 and 24/25, respectively. The cells were transfected for 48–72 h using siPORT lipophilic transfection agent with the two siRNA, either alone or in combination at 125–150 \( \mu \)M.

**Migration Assay of \( \alpha 2 \) Knockdown Endothelial and Fibrosarcoma Cells—**HUVEC and HT1080 cells were transfected with the optimal concentrations of siRNA, and the time span was analyzed by immunoblotting. HUVEC and HT1080 that were treated with siPORT alone were used as a control. Both control and \( \alpha 2 \) siRNA-treated cells were preincubated with various concentrations of endorepellin for 30 min prior to migration through a collagen I-coated polycarbonate membrane, with 8- \( \mu \)m pores in a 48-well Boyden chamber (Neuroprobe Inc., Gaithersburg, MD). About 8 × 10\(^3\) HUVEC or HT1080 cells were loaded into the upper chamber and allowed to migrate at 37°C with 5% CO\(_2\) for 4–6 h. VEGF\(_{165}\) (R & D Systems, Minneapolis, MN) at a concentration of 10 ng ml\(^{-1}\) in the lower chambers as a chemo-attractant for HUVEC, and heat-inactivated medium conditioned by HT1080 as a chemo-attractant for HT1080.

**Immunofluorescence Analysis of \( \alpha 2 \beta 1 \) Knockdown Cells—**HUVEC and HT1080 cultured for 24 h on lysine-treated eight-well slides coated with rat tail collagen I (100 \( \mu \)g ml\(^{-1}\)) (BD Biosciences) were transfected with the two validated \( \alpha 2 \) siRNA constructs (150 \( \mu \)m each). The cells were rinsed with PBS, fixed in 5% paraformaldehyde, and permeabilized with 0.1% Triton X-100 for 10 s. The cells were rinsed in PBS and then blocked with 5% BSA in PBS for 1 h. Polyclonal anti-\( \alpha 2 \) primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to wells at a 1:50 dilution in 5% BSA/PBS for 1 h. Primary antibody was detected using a fluorescein isothiocyanate-conjugated rabbit anti IgG (1:200) (Santa Cruz Biotechnology). The nuclei were visualized using DAPI. All of the images were acquired on an Olympus BX51 microscope equipped with a SPOT camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

**Genotyping of Integrin \( \alpha 1 \) and \( \alpha 2 \) Null Mice and Isolation of Lung Microvascular Endothelial Cells—**Tail genomic DNA was subjected to PCR in 20 \( \mu \)l of reaction volume with 1.25 mM MgCl\(_2\) and 1 pmol ml\(^{-1}\) each primer using a mix of three primers. Primer sequences were as follows: \( \alpha 1 \)B1+/+5’-ggttggctgcatcaaggattc-3’, \( \alpha 1 \)B1+/−5’-ggacaccctcgaggtcggggg-3’, \( \alpha 1 \)B1−/−5’-aagttgctcgcttgctcta-3’. \( \alpha 1 \)B1 common, 5’tttcacaccataaatggtcgc-3’, \( \alpha 2 \)B1+/+5’-ctttgaactgatcttgg-3’, \( \alpha 2 \)B1+/−5’-ccttgctatgctaggcttc-3’, \( \alpha 2 \)B1−/−5’-tggtttttctctcctgag-3’, and \( \alpha 2 \)B1 common, 5’-aagttgctcgcttgatc-3’.

Lung microvascular endothelial cells were isolated from wild-type or integrin null mice as previously described (25). Briefly, the lung vasculature was perfused with PBS, 2.5 mM EDTA followed by 0.25% trypsin, 2.5 mM EDTA via the right ventricle. The lungs were removed and incubated at 37°C for 20 min. The visceral pleura was subsequently trimmed, and the perfusion was repeated. Primary endothelial cells were recovered and grown in EGM-2-MV containing 5% fetal calf serum (Clonetics). The cells at passages 2–4 were used for the experiments.

**Syngeneic Tumor Xenografts and Quantification of Tumor Angiogenesis—**Female integrin \( \alpha 2 \)B1+/+ and \( \alpha 2 \)B1+/− mice in C57Bl/6 background were injected subcutaneously with 10\(^6\) LLC cells. On the day tumors became visible, the mice were randomized into two groups, and one group received intraperitoneal injections of human recombinant endorepellin, whereas the other received vehicle (PBS) alone (20). Tumor sectioning and immunostains were performed as previously described.
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(20). The following antibodies were used: rat anti-mouse CD31 monoclonal antibody (BD, Biosciences, San Jose, CA) and mouse anti His6 monoclonal (ABM, Vancouver, Canada). Both antibodies were diluted 1:100. The epitopes were revealed by using fluorescein isothiocyanate-conjugated goat anti rat IgG and rhodamine-conjugated goat-anti mouse IgG (Santa Cruz Biotechnology) secondary antibodies. Sections were counterstained with DAPI. Sections from three different tumors in each group were stained with CD31, and pictures were taken at 20× magnification to cover the entire tumor section with a fixed exposure of 500 ms. The acquired images (n = 30–50) were quantified using the Image J software package (National Institutes of Health, Bethesda, MD).

All of the statistical analyses were carried out with SigmaStat for Windows version 3.10 (Systat Software, Inc., Port Richmond, CA). The results were compared by using the two-sided Student’s t test, and the differences were considered statistically significant at p < 0.05.

RESULTS

The Soluble α2β1 Integrin Ectodomain Interacts Specifically with Endorepellin in a Cation-independent Manner—To investigate the molecular interaction between endorepellin and the α2β1 integrin, we utilized a soluble heterodimeric α2β1 integrin. This soluble receptor consists of the ectodomain of both α2 and β1 integrin subunits noncovalently associated by the dimerizing motif of the two transcription factors Fos and Jun, respectively (21). This soluble integrin binds with high affinity collagen types I, II, and IV as the native integrin and has been successfully utilized in various protein/protein interaction assays (21). Using this approach, together with a battery of monoclonal antibodies against the α2 I domain, the precise mapping of the disintegrin rhodocetin, an RGD-independent ligand of the α2β1 integrin, on the α2 I domain was determined (22). We conducted several binding studies using endorepellin and the soluble α2β1 integrin. Unexpectedly, when endorepellin was utilized as the immobilized ligand, there was no significant binding to the soluble α2β1 (Fig. 1A). Under identical experimental conditions, immobilized collagen I readily bound to the soluble α2β1 receptor, and this interaction was enhanced by the activating monoclonal antibody 9EG7 and abolished by the addition of EDTA (Fig. 1A). In contrast, when endorepellin was used as a soluble ligand, it significantly bound to the immobilized α2β1 (Fig. 1B). Surprisingly, binding levels were found to be identical in the presence or absence of EDTA. As an internal positive control, we used the CB3 fragment of collagen IV, which harbors the α2 I domain-binding site (23, 24). The binding of CB3 [IV] to immobilized α2β1 receptor was robust and significantly reduced by EDTA (Fig. 1B).

When a range of concentrations of endorepellin was added to α2β1-coated plates in the presence of Mn2+ (1 mm) or EDTA (10 mm), binding curves were almost identical, confirming the cation-independent interaction (Fig. 1C). Next, we determined whether endorepellin/α2β1 interaction was mediated by the α2 I domain, the region of the integrin known to bind collagen (26). When increasing concentrations of soluble endorepellin were added to the microtiter plates at the concentrations shown (Fig. 1D), a saturable binding of endorepellin to the α2 I domain was observed, albeit at lower levels than the whole heterodimeric integrin ectodomain (Fig. 1D) in agreement with our previous studies (14).

Functional Block of Endorepellin Activity by Soluble α2β1 Integrin and Its α2 I Domain—Having established a physical interaction between the soluble α2β1 integrin and endorepellin, we next wished to prove whether this interaction would have a biological meaning using live endothelial cells. Utilizing a highly sensitive functional assay (14), we found that actin stress fiber disassembly evoked by a short exposure to endorepellin could be almost completely abolished by preincubating endorepellin with either soluble α2β1 or the α2 I domain (Fig. 2, A–F). Notably, similar effects were obtained using the terminal LG module, LG3, of endorepellin (supplemental Fig. S1), which is the active
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binding site for the integrin (19). To further corroborate these finding, we quantified the total number of attached cells, as an adhesion assay on collagen I. The number of adherent endothelial cells was drastically ($p < 0.001$) reduced when incubated with endorepellin (Fig. 2G). However, this effect was abrogated by the soluble ectodomain of the $\alpha_2\beta_1$ integrin and significantly reduced ($p < 0.05$) by preincubation with the $\alpha_2$ I domain (Fig. 2G). Collectively, these findings prove that the physical interaction between endorepellin and soluble $\alpha_2\beta_1$ integrin ectodomain translates into a functional block of endorepellin angiostatic activity on endothelial cells.

Integrin $\alpha_2\beta_1$ Is Vital for Normal Migration of Endothelial and Fibrosarcoma Cells through Collagen I—To further investigate the functional role of $\alpha_2\beta_1$integrin, we took a gene targeting approach using validated siRNA to knockdown the $\alpha_2$ subunit in HUVEC and HT1080, a cell line that depends on $\alpha_2$ as the main collagen I receptor (21). Immunoblotting of cell lysates revealed almost complete knockdown of the integrin expression in HUVEC (~5% of control levels) when the cells were transfected with two siRNA constructs each at a concentration of 150 nM targeting different regions of the $\alpha_2$ mRNA (Fig. 3A). Similarly high levels of knockdown were also seen in HT1080 transfected at 50 and 100 nM concentrations (Fig. 3B). The results were confirmed by immunofluorescence analysis that showed an almost complete loss of the $\alpha_2$ signal in the transfected HUVEC and HT1080 as compared with controls (Fig. 3, C and D, respectively).

Migration of HUVEC through collagen I was used as a test for functionality because this is a critical process required for angiogenesis in vivo (27). Knockdown of the $\alpha_2$ subunit caused a profound inhibition of migration, especially at higher dosages (Fig. 3E). The inhibition of HUVEC migration in the cells deficient in $\alpha_2\beta_1$ was equal to that observed by endorepellin treatment, suggesting that this receptor is crucial for migration of endothelial cells. This further stresses the importance of this integrin in the migratory ability of HUVECs in vitro. However, in vivo, the lack of $\alpha_2\beta_1$ integrin (see below) did not delay the process of tumor angiogenesis, a phenomenon that has also been observed in mutant mice lacking other integrins (28).

Genetic Requirement of $\alpha_2\beta_1$ for Endorepellin-mediated Angiostatic Activity—Next, we conducted in vitro angiogenic assays utilizing lung microvascular endothelial cells isolated from wild-type or mutant animals lacking either the $\alpha_1$ (25) or the $\alpha_2$ (29) subunit and thus lacking functional $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrin receptors. The genotype of the wild-type and the two genetically engineered mice is shown in Fig. 4A. Wild-type cells showed the highest levels of VEGF-driven migration through collagen I and displayed a complete inhibition of migration when preincubated with endorepellin (Fig. 4B). Under unchallenged experimental conditions, both $\alpha_1\beta_1^{-/-}$ and the $\alpha_2\beta_1^{-/-}$ endothelial cells showed a reduced migration as compared with wild-type cells (Fig. 4B). However, $\alpha_2\beta_1^{-/-}$ endothelial cells showed a greater decline in migratory ability than the $\alpha_1\beta_1^{-/-}$ cells (45% versus 30%). These data are in agreement with siRNA experiments shown above and suggest that the $\alpha_2\beta_1^{-/-}$ integrin is a key receptor for VEGF-induced chemotactic migration, a main biological process in angiogenesis (27, 30).

Most importantly, only the wild-type and the $\alpha_1\beta_1^{-/-}$ microvascular endothelial cells showed a complete inhibition of migration when preincubated with endorepellin, whereas the $\alpha_2\beta_1^{-/-}$ endothelial cells were totally unresponsive (Fig. 4B). These data provide robust genetic evidence that the inhibition of migration caused by endorepellin requires the presence of the $\alpha_2\beta_1$ integrin receptor.

Next, we tested the wild-type and mutant endothelial cells in actin disassembly assays (14). Although endothelial cells from wild-type and $\alpha_1\beta_1^{-/-}$ mice responded well to endorepellin by showing rapid actin disassembly, the endothelial cells from $\alpha_2\beta_1^{-/-}$ mice were totally unresponsive to endorepellin (Fig. 4C). Notably, the same drastic disassembly of stress...
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FIGURE 3. Integrin α2β1 is vital for proper migration of endothelial and fibrosarcoma cells through collagen I. A and B, immunoblottings of HUVEC (A) and HT1080 (B) transfected with two validated α2 siRNA constructs at the shown concentrations. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Notice the marked suppression of the α2 subunit expression in both cases. C and D, immunofluorescence analysis of control and siRNA-treated cells using anti-α2 integrin antibody. Notice the significantly reduced staining for the integrin (green) in the siRNA-treated endothelial (C) and HT1080 (D). Bar, 40 μm. E, reduced endothelial cell migration through collagen I following transfection with two validated α2 siRNA constructs at concentrations shown. The bottom chamber included VEGF165 (10 nM) as a chemo-attractant. F, reduced migration through collagen I of HT1080 transfected with two α2 siRNA constructs (150 nM each) for 120 h and preincubated with increasing concentrations of endorepellin. Migration was allowed to occur in serum-free conditions for 5 h using heat-inactivated media conditioned by HT1080 as chemo-attractant. The values represent the means ± S.E. (n = 3).

DISCUSSION

Using an integrated experimental strategy employing a soluble form of the α2β1 integrin, endothelial cells deficient in the integrin α1 or α2 subunits, siRNA knockdown of the integrin α2 subunit, and syngeneic tumor xenografts growing in mice lacking the α2β1 integrin receptor, we provide new biochemical and genetic evidence that the central perlecan receptor on endothelial cell is the α2β1 integrin. We further discovered a novel cation-independent binding between endorepellin and integrin α2 I domain and showed that integrin α2β1 is necessary for the recruitment of endorepellin to the vasculature and for its anti-angiogenic properties both in vitro and in vivo. The strategic topography of perlecan and its ability to modulate various growth factors and morphogens (4–6, 31–35) renders perlecan a key factor not only in the proper assembly and homeostasis of basement membranes but also in regulating angiogenesis during development and cancer growth.
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To gain insights into endorepellin/α2β1 interaction, we utilized a recombinant integrin that harbors the ectodomain of both α2 and β1 subunits noncovalently linked by the dimerizing (zipper) motifs of the two transcription factor Fos and Jun (21). This soluble heterodimeric integrin ectodomain is fully functional and, similar to the parent trans-membrane receptor, requires divalent cations and binds collagens I, II, IV, and V (36). We discovered that endorepellin bound to the α2β1 ectodomain only when provided as a soluble ligand but failed to be recognized by the integrin when it was presented as an immobilized ligand. This is likely due to the fact that endorepellin is highly sensitive to denaturation and loses its three-dimensional configuration when dried on the microtiter plates. Similarly, we found that endorepellin needed to be in solution to properly interact with the integrin α2 I domain using Biacore technology (14). We also found that endorepellin, in contrast to collagen CB3[IV], binds to the α2β1 integrin via the I domain in a cation-independent manner. The crystal structure of the α2 I domain bound to the homotrimeric GFOGER collagen peptide has demonstrated the requirement for metal ions as bridging cofactors for collagen binding (26, 37). Collagen binding induces a conformational change within the α2 I domain that is transferred to the rest of the integrin ectodomain and eventually to the cytoplasmic tail, thereby triggering intracellular signals (38). However, noncollagenous and RGD-independent ligands for the α2β1 integrin, such as human echovirus-1 (39), MMP-1 (40), and rhodocetin (21, 22), do not require divalent cations to bind the α2 I domain. Thus, endorepellin belongs to this class of molecule that possess an RGD-independent and cation-independent binding affinity for the α2β1 receptor (36).

The most C-terminal portion of endorepellin, LG3, possesses most of endorepellin angiostatic properties (14, 19). LG modules can bind, in different proteins, a wide diversity of ligands including Zn$^{2+}$, Ca$^{2+}$, steroids, glycans, proteoglycans, and proteins (41–43). Laminin α2LG4 and LG5, despite possessing only 24% sequence identity (much like LG3-laminin α2LG5 alignment in endorepellin), superimpose structurally with Ca$^+$ root mean square deviation of only 1.1 Å, using 139 of 181 Cos for the superposition (19). The predicted structural similarity among LG modules and known crystal structures of prototypical LG domains (44, 45) allowed us to construct a comparative model of endorepellin LG3 that consists of a β-sandwich or jellyroll composed of 14 antiparallel β strands arranged in two sheets similar to the C-type lectins (19). The shared function of endorepellin and snake venom proteins, such as rhodocetin (22) and EMS16 (45, 46), in specifically targeting the α2 I domain of the integrin might be based on their structural commonality because they all share a jellyroll structural typical of C-type lectins.

In knockdown experiments targeting the α2 subunit of the receptor with two validated siRNA, we found that loss of the integrin significantly impaired the ability of endothelial and fibrosarcoma cells to migrate through collagen, a key event for tumor angiogenesis and remodeling (47). The inhibition of endothelial cell migration in the cells deficient in α2β1 was
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FIGURE 5. Integrin α2β1 is the in vivo functional receptor for endorepellin. A, growth curve of LLC tumor xenografts in wild-type and integrin α2β1−/− mice (n = 5/group) treated with or without human recombinant endorepellin. Endorepellin shows no effect on reducing the tumor growth in mice carrying a targeted deletion of the integrin α2 subunit and thus having no functional α2β1 receptor. B, photomicrographs of the tumor surface from representative LLC tumor xenografts from the three groups of A. Notice that the tumor xenografts growing into the α2β1−/− mice are well vascularized and larger than those growing in the same genetic C57Bl/6 background but possessing the α2β1 integrin. C, immunofluorescent images of tumor cryosections from mice treated with either vehicle (PBS) or recombinant endorepellin, as indicated. The sections were stained with a rat monoclonal antibody recognizing the endothelial cell-specific marker CD31 (green) for detection of the vasculature. Notice that endorepellin has little effect on reducing angiogenesis in tumors lacking the α2β1 integrin, whereas tumors from the wild-type animals are clearly less vascularized. Bar, 200 μm. D, quantification of blood vessel density in LLC xenografts growing in the three genetic backgrounds as indicated. The values represent the means ± S.E. (n = 30–50). ***p < 0.001. E, immunofluorescent images of LLC xenografts showing the highly selective and specific targeting of endorepellin (red) to the tumor vasculature (green) in the wild-type tumor xenografts but not in those derived from α2β1−/− mice. Cryosections were reacted with anti-CD31 to label endorepellin; the nuclei are stained with DAPI (blue). Bar, 200 μm.

equal to that observed by endorepellin treatment, suggesting that this receptor is crucial for migration of endothelial cells. Seemingly, the fibrosarcoma cells devoid of the α2β1 integrin subunit showed a ~75% inhibition of migration, and this inhibition was not augmented by increasing endorepellin concentrations. Keratinocytes derived from the α2β1−/− also exhibit impaired adhesion to collagen I, whereas adhesion to laminin 111 is indistinguishable from wild-type cells (48).

By utilizing two different mutant mice, one lacking the α2 and the other lacking the α1 integrin subunit, we showed that the effects of endorepellin on migration and cytoskeletal disassembly on microvascular endothelial cells isolated from these animals are mediated via the α2β1 and not the α1β1 integrin. In agreement with the knockdown results, lung microvascular endothelial cells lacking the α2β1 integrin showed ~45% reduction in VEGF-mediated migratory ability, whereas those lacking the α1β1 showed ~30% reduction. The apparent discrepancy in their ability to migrate through collagen I between mouse microvascular endothelial cells lacking α2β1 (~45% reduction) and the HUVEC treated with the siRNA against the integrin (80–90% reduction) can be partially reconciled by the fact that in HUVEC the α2β1 integrin is the principal functional collagen receptor, and only low levels of the α1β1 integrin are present (49, 50). In contrast, it has been shown that microvascular endothelial cells express both integrin receptors and that α1β1 and α2β1 integrins provide critical support for VEGF signaling, endothelial cell migration, and tumor angiogenesis (30, 51, 52). Thus, it is likely that during development α1β1 might compensate for the absence of α2β1, thereby allowing the lung microvascular endothelial cells to maintain about half of their migratory ability.

Using syngeneic LLC xenografts growing in a defined genetic mouse background possessing or lacking the α2 integrin subunit, we observed an endorepellin-mediated inhibition of tumor angiogenesis and growth only in the α2β1+/+ background. Moreover, systemically delivered endorepellin targeted the tumor vasculature only in the α2β1+/+ background. This provides a strong genetic evidence of in vivo α2β1 requirement for the functional activity of endorepellin.

Despite the in vitro absolute requirement for the α2β1 integrin, the process of in vivo tumor angiogenesis was not delayed in the α2β1−/− mice integrin; the syngeneic LLC tumor xenografts showed robust angiogenesis and grew as well or slightly better in the α2β1−/− than the wild-type background. This phenomenon has also been observed in mutant mice lacking other integrins (28) and in two animal models of wound healing utilizing α2β1−/− in the same C57Bl/6 background as in the present study (53, 54). In the case of the α2β1−/− mice, the vascularization of the wounded skin was significantly increased and postulated to be due to either failure of endogenous endorepellin to bind α2β1 and exert its angiostatic effects or due to a shift toward α1β1 integrin, which is known to mediate potent pro-angiogenic stimuli (25). Additional mechanisms of action that could contribute to the enhanced angiogenesis in the α2β1−/− mice include overexpression of other pro-angiogenic integrins and cross-talk between integrin α2β1 and other integrins and growth factor receptors, causing trans-dominant inhibition of those receptors when α2β1 is present. This was one hypothesis for the increased pathological angiogenesis in integrin β3 and β5 null animals (55). Finally, the integrin α2β1 null animals have an enhanced expression of various matrix metalloproteases during wound healing (53).
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Suppression of perlecan gene expression by antisense RNA inhibits tumor growth and angiogenesis in a variety of tumor cell systems including melanomas, colon, and prostate carcinomas (56–59). However, we found that HT1080 fibrosarcoma xenografts behaved in the opposite way; tumor growth and angiogenesis was enhanced by antisense perlecan expression (60). These discordant results could now be reconciled on the basis of the new evidence reported here. It is possible that lack of perlecan secreted by the fibrosarcoma cells removes endogenous endorepellin, thereby allowing the tumor xenograft to grow more vascularized. The unique dependence of HT1080 on the α2β1 integrin makes this argument even more compelling.

In conclusion, by using a genetic approach coupled with biochemical and functional assays, we have shed new light on the mechanism of action of endorepellin in mediating its anti-angiogenic properties both in vitro and in vivo. This unique integrin ligand, which has been conserved in evolution for millions of years, might represent a future class of therapeutic proteins that may prove beneficial in the management of tumor angiogenesis, either alone or in combination with other therapies.

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