Endorepellin, a Novel Inhibitor of Angiogenesis Derived from the C Terminus of Perlecan*

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Perlecan, a ubiquitous basement membrane heparan sulfate proteoglycan, plays key roles in blood vessel growth and structural integrity. We discovered that the C terminus of perlecan potently inhibited four aspects of angiogenesis: endothelial cell migration, collagen-induced endothelial tube morphogenesis, and blood vessel growth in the chorioallantoic membrane and in Matrigel plug assays. The C terminus of perlecan was active at nanomolar concentrations and blocked endothelial cell adhesion to fibronectin and type I collagen, without direct binding to either protein; henceforth we have named it “endorepellin.” We also found that endothelial cells possess a significant number of high affinity ($K_d$ of 11 nM) binding sites for endorepellin and that endorepellin binds endostatin and counteracts its anti-angiogenic effects. Thus, endorepellin represents a novel anti-angiogenic product, which may retard tumor neovascularization and hence tumor growth in vivo.

Perlecan is a modular proteoglycan that participates in the formation and maintenance of basement membranes in various organs (1–5). The protein modules of perlecan have striking homology to polypeptides involved in lipid uptake, growth control, cell-cell interactions, and adhesion (6–8). Its highly refined molecular architecture, coupled with its ubiquity, suggests that perlecan may play key biological functions during ontogeny, tissue remodeling or transformation (9, 10). Lack of perlecan causes embryonic lethality with severe cephalic and cartilage abnormalities (11, 12). Although basement membranes can develop in the absence of perlecan, the majority of the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent and the perlecan-deficient mice succumb to intrapericardial hemorrhages at day 10.5, when vasculogenesis is prominent

1 The abbreviations used are: FGF2, fibroblast growth factor 2; LG, laminin-G like module; CAM, chorioallantoic membrane; HUVEC, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; BSA, bovine serum albumin; AP, alkaline phosphatase; HA, hemagglutinin; NTA, nitritolactric acid; ELISA, enzyme-linked immunosorbent assay; BS3, bis-sulfosuccimidyl-suberate.
the yeast two-hybrid system, we identified collagen XVIII, including the anti-angiogenic factor endostatin, as a strong candidate. We discovered that the C terminus of perlecain, henceforth named “endorepellin,” counteracted the anti-angiogenic effects of endostatin, while by itself potently inhibited four adhesion domains of various substrata, including fibronectin and type I collagen. Moreover, endothelial cells possess a significant number of high affinity sites for endorepellin, which could be cross-linked with bis-sulfosuccimydyl-suberate (BS3) to form high $M_c$ complexes. Thus, endorepellin may represent a novel anti-angiogenic tool against cancer.

**EXPERIMENTAL PROCEDURES**

**Cells, Yeast Two-hybrid Screening, and Co-immunoprecipitation**—Primary cultures of HUVECs were prepared from fresh umbilical cords and cultured on gelatin-coated flasks in M199 or M200 media (Invitrogen) supplemented with 10% fetal bovine serum, 50 µg/ml heparin, and endothelial cell growth supplement, isolated from bovine hypothalami. Only passages 4–8 were used. A431 squamous carcinoma, HeLa squamous carcinoma, HT1080 fibrosarcoma, WiDr colon carcinoma, MCF7 breast carcinoma, and M2 mouse melanoma cells were obtained from the American Type Culture Collection (Manassas, VA). We employed the Matchmaker GAL4 two-hybrid system 3 (Clontech, Palo Alto, CA), which adopts three independent reporter genes (His, Ade, and either α- or β-galactosidase) for selection. Endorepellin, subcloned into the pG-BKT7 vector, was used as bait to screen a keratinocyte library constructed in the pACT2 vector. The clones growing in selective medium were replated in quadruplicate minus plates containing X-gal. Interacting cDNAs were identified by systematic sequence retrieval. Recombinant mutants were generated by PCR using oligonucleotides, which included suitable restriction sites to allow unidirectional ligation into the pG-BKT7 vector (38). Various constructs were in vitro transcribed and translated in the presence of [35S]methionine (ICN Pharmaceuticals, Costa Mesa, CA) using the TnT™ reticulocyte lysate system (Promega, Madison, WI). Aliquots were co-immunoprecipitated with affinity-purified, anti-hemagglutinin (αHA) rabbit polyclonal antibodies (Clontech). The immune complexes were captured with protein A/G-agarose beads (Pierce), washed with 10 mM Hepes pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1% glycerol, 200 mM Na$_2$VO$_4$, 20 mM NaF, and a protease inhibitor mixture (Roche Diagnostics GmbH, Mannheim, Germany), and separated in polycrylamide gels. The gels were fixed in ethanol/glacial acetic acid, incubated for 20 min with AMPLIQON’s Bioindex, and processed for light microscopy. The newly formed blood vessels present in the Matrigel plug were counted as described above.

**Endothelial Cell Migration, Tube Formation, and Chorioallantoic Membrane (CAM) Assays**—A 48-well Boyden chamber (Neuroprobe Inc., Gaithersburg, MD) was used for HUVEC migration assays with VEGF$_{165}$. R&D Systems, Minneapolis, MN) as a chemotactant. HUVECs migrated through 8-µm nuclopre peptide, polyvinylpyrrolidone, or polylysine-coated inserts (Corning, Cambridge, MA) precoated for 48 h with 100 µg ml$^{-1}$ collagen type I (Collaborative Biomedical Products, Bedford, MA). About 2 × 10$^5$ HUVECs were preincubated for 1 h with different concentrations of endorepellin and/or endostatin from F. chinensis (Calbiochem-Novabiochem, San Diego, CA), and allowed to migrate through the filter for 6 h at 37 °C in 5% CO$_2$, with or without 10 ng ml$^{-1}$ VEGF$_{165}$. After 6 h, the lower chambers were washed, fixed, stained with Diff-Quick stain (VWR Scientific Products, Bridgeport, NJ), and the transmigrated cells were counted using conventional microscopy. For in vitro tube-like formation, 4 × 10$^5$ HUVECs were seeded for 18 h onto 12-well dishes coated with 100 µg ml$^{-1}$ collagen type I and then covered with a second layer of collagen gel (39). Cultures were incubated until gels solidified, typically 15–30 min, and then given 1 ml of serum-free media with the various test agents and control substances. For the CAM assay, fertilized White Leghorn chick eggs were incubated at 37 °C. After 3 days of incubation, 3–ml of albumin were removed to detach the CAM, and a small square window was formed. The window was then sealed with tape, and the eggs were returned to the incubator. The CAMs were removed under sterile conditions and placed on the CAMs, and various test factors were applied. In addition, CAM assays were performed using sponges containing either 0.5 × 10$^5$ WiDr colon carcinoma cells alone or in combination with 3 µg of recombinant endorepellin. Three-day-old embryos (n = 20) were used in each experiment. The mean vessel area was calculated using the NIH Image software program (version 1.61) at least using four embryos per experimental point. Ten squares of ~500 µm$^2$ each were randomly selected around the sponge area and digitized. The background was uniformly adjusted so that it would appear white, whereas the vessels would be black. The pixel density of the vascularized areas was measured, and the values were normalized relative to the area (µm$^2$). Student’s $t$-test was used to compare the values of the experimental and control samples. A value of $p < 0.05$ was considered as statistically significant. Matrigel plug assays were essentially performed as previously described (40). Briefly, 100 µl of Matrigel (BD PharMingen, San Diego, CA) containing FGF2 (10 ng/animal), in the presence of or absence of endorepellin (12 µg/animal), was injected into the dorsal subcutaneous regions of 10–12 nude mice. Mice were sacrificed 2 weeks after the injection, and the skin was removed to analyze the blood vessel formation. The skin samples were photographed, fixed in buffered formaldehyde, and processed for light microscopy. The newly formed blood vessels present in the Matrigel plug were counted as described above.

**Dithiothreitol Cross-linking**—Endorepellin (10 µg) was labeled to high specific activity (10$^{10}$ cpm mol$^{-1}$) using iodoagen-dosed tubes (Pierce). For saturation binding and Scatchard analysis, confluent HUVECs in 24-well plates were incubated with increasing concentrations of 125I-endorepellin for 2.5 h at 4 °C in M199 containing 0.1% BSA, washed several times, and extracted in the presence of protease inhibitors (41). Estimates of receptor affinity and total binding capacity were made with Sigma Plot 5.0 software. For covalent affinity cross-linking, HUVECs were incubated with various concentrations of 125I-endorepellin for 2 h, and then incubated for 30 min at 4 °C with 20 µM BS3, a membrane-impermeable cross-linker. After termination of the reaction with 10 ml Tris, pH 7.5, the cells were then washed, suspended in 0.3 M sucrose, 10 m€l$^{-1}$ homobiotin, and incubated at 4 °C for 3 h, washed three times, and used for immunoblotting with anti-receptor antibodies (37). The 125I endorepellin signal was assayed by a colorimetric test (36). The anti-adhesive assays were performed in a similar way on fibronectin-coated plates. After blockage with 1% BSA, the cells were added to the wells in the presence of

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increasing concentration of endorepellin or endostatin. After 1 h of incubation, the wells were treated as above.

Expression of Endorepellin/Alkaline Phosphatase Chimeric Protein and Binding Studies—The heat-stable human placental alkaline phosphatase (AP) was amplified by PCR from our previously described construct (42) and ligated in-frame onto the C terminus of endorepellin in the pCEP-Pu vector. The construct was electroporated into 293EBNA cells as described above. Following several weeks in selective medium (250 μg ml⁻¹ G418 and 500 μg ml⁻¹ puromycin), several endorepellin/AP-expressing clones were identified using the Great EscAPE™ SEAP system (Clontech), which detects AP. Briefly, conditioned media from untransfected cells (negative control) and from stably transfected cells secreting either AP (positive control) or endorepellin/AP chimera were incubated at 65 °C for 30 min to inactivate endogenous phosphatases, cooled on ice, and then mixed with CSPD substrate/chemiluminescent enhancer for 10 min, followed by exposure to x-ray film for 5–10 s. In addition, the nature of the AP alone and the chimeric protein was identified following immunoprecipitation with a mouse monoclonal antibody (Clone 8B6, Sigma) against human placental AP linked to agarose beads. Binding studies were performed using various cell lines incubated with 0.5 ml of serum-free media conditioned by expressing or control 293-EBNA cells for 48 h. After a 1.5-h incubation at 25 °C, the cells were washed six times, lysed in 1% Triton X-100, 20 mM Tris-HCl, pH 7.5, and processed as stated above.

RESULTS

Endorepellin Is a Novel Interacting Partner for Perlecan Domain V/Endorepellin—To discover novel interacting partners for perlecan protein core we utilized the entire domain V of perlecan (7, 8), which we named endorepellin (amino acids 3687–4391, Fig. 1a) as bait and screened a keratinocyte cDNA library in the yeast two-hybrid system. This domain consists of three laminin-type G (LG1-LG3) modules separated by four EGF-like (EG1-EG4) modules, in an arrangement highly conserved across species (2, 43). One of the strongest interacting clones (clone A3) encoded the C-terminal half of collagen type XVIII, including the NC1 domain containing the potent antiangiogenic factor endostatin (Fig. 1b). Because endostatin inhibits endothelial cell proliferation and effectively arrests the growth of several tumors (44), and because perlecan and endostatin co-localize in most basement membranes (2, 3, 45, 46), we reasoned that an interaction between these two proteins could occur in vivo and could play a role in tumor progression. Therefore, we subcloned the collagen fragment into the pGADT7 vector, and the interaction with endorepellin was once more tested with the yeast two-hybrid system on a one-to-one basis. The growth of the cells in quadruple minus medium was comparable to that of the positive control (pGBK7-T53/pGADT7-T), as well as the blue color generated by α-galactosidase expression (Fig. 1c). To corroborate the yeast interaction, we in vitro transcribed and translated endorepellin and collagen XVIII (clone A3), showing the ~81- and ~65-kDa fragments, respectively (Fig. 1d). We could co-precipitate the two proteins with an anti-HA antibody that recognizes the oligopeptide epitope HA present at the C terminus of collagen XVIII (Fig. 1d). To determine whether endostatin, which is encoded by the C terminus of collagen type XVIII (Fig. 1b), could interact with endorepellin, we cloned the endostatin sequence into pGADT7 vector, and then in vitro transcribed and translated the insert, which generated a 23-kDa band (Fig. 1e, lane 3). As a further control, we subcloned domain III of perlecan into the pGBK7 vector and then in vitro transcribed and translated the insert, which gave the expected ~130-kDa peptide (Fig. 1e, lane 1). The results showed that only endorepellin interacted with endostatin (Fig. 1e, lane 5). In contrast, domain III of human perlecan did not bind (lane 4).

To establish a direct interaction between endorepellin and endostatin, we performed several solid-phase binding assays using 125I-labeled endorepellin as the soluble ligand, and endostatin, fibronectin or collagen I as the solid substrates coated onto Immulon wells. In these experiments, recombinant endorepellin and endostatin were generated in 293-EBNA cells (see below), and the former was radiiodinated to reach specific activities of ~10⁹ cpm mol⁻¹. We found a saturable binding of 125I-labeled endorepellin to endostatin in the 60–70 nM range, with half maximal binding of ~48 nm (Fig. 1f). Specificity of binding was determined by competition experiments with 25-molar excess of cold endorepellin (Fig. 1f). In contrast, endorepellin did not substantially bind to either fibronectin or collagen type I (Fig. 1g).

Endostatin Interacts Specifically with the LG2 Module of Endorepellin—To establish the precise location of this interaction, we generated seven deletion mutants (Δ1–Δ7) of domain V/endorepellin (Fig. 2). Robust growth in quadruple minus media was observed in cells co-transformed with full-length endorepellin and Δ1 and Δ5, the only two mutants that encompassed the LG2 module (Fig. 2a). These results were corroborated by α- and β-galactosidase assays (Fig. 2b). In addition to growth in amino acid-deficient media, transcription of LacZ (α- and β-galactosidase) under the control of distinct GAL4 upstream-activating sequences, and the subsequent ability of the co-transformant yeast strains to express functional galactosidase activity, provides an additional strong proof of a true protein-protein interaction (47). Thus, the LG2 module of endorepellin is likely to be the specific site of endostatin binding, although in the native perlecan core protein, the role of the flanking sequences remains to be established.

Recombinant Endorepellin Is Anti-angiogenic—Human recombinant endorepellin, generated in 293-EBNA cells, migrated on SDS-PAGE as a single band of the predicted ~81 kDa, whose identity was further confirmed by immunoblotting with anti-His6 antibody (Fig. 3a) and ELISA using a specific monoclonal antibody against domain V (17) (not shown). Because murine perlecan domain V can be substituted with glycosaminoglycan side chains (37, 48), we subjected 10 μg of purified endorepellin to DEAE Sephadex chromatography. None of the purified endorepellin bound to the DEAE column under relatively low salt (NaCl, 150 mM) conditions (Fig. 3b, lane 3) indicating that the human preparation did not contain glycosaminoglycan side chains. Interestingly, our construct behaves like the Drosophila perlecan domain V, which when expressed in the same 293-EBNA cells, migrates as a single band without any overt glycanation (49).

To test the biological properties of endorepellin, we utilized VEGF-induced migration of HUVEC to passages 4–8 (50). It is well established that the motility and vectorial migration of endothelial cells that coincidently occur with invasion, are fundamental components of angiogenesis (33, 51, 52). When VEGF was used in the lower chamber, there was a complete suppression of HUVEC migration through the membrane at 1–10 μg ml⁻¹ (12–120 nM) endorepellin (Fig. 3c). Interestingly, endorepellin was more active than recombinant endostatin purified from Pichia pastoris yeast cells. Subsequent dilution experiments revealed that endorepellin was fully active at 0.5 μg ml⁻¹ (6 nM) (Fig. 3d), with a calculated IC₅₀ of 2 nM (~0.1, n = 11). In some preparations, endorepellin was active even at picomolar concentrations (not shown). These experiments were repeated several times with various preparations of endorepellin, and a marked suppression of HUVEC migration was consistently found. In contrast to endostatin, the migratory response was not dependent on the preincubation of the endothelial cells with endorepellin. In experiments where endorepellin was placed in the lower chambers of the invasion assay, we found similar inhibition of VEGF-induced migration (not shown).
Next, we investigated whether the inhibition of HUVEC migration could lead to a decreased angiogenesis in vivo. Using the chorioallantoic membrane (CAM) assay, we discovered that endorepellin significantly reduced the angiogenic activity of VEGF (Fig. 4). In the presence of VEGF, the characteristic spoke wheel-like vessel formation was induced toward the sponge (Fig. 4a). In the presence of endorepellin (Fig. 4b), the vessel sprouts were markedly reduced to a level comparable to the negative control (Fig. 4c).

Next, we tested whether endorepellin could counteract the angiogenic stimuli of WiDr, a highly tumorigenic colon carcinoma cell line (53). Indeed, long term culture of capillary endothelial cells was originally obtained by culturing endothelial cells with media conditioned by malignant cells (54). This indicates that tumor cells express a large repertoire of growth-promoting factors that support endothelial cell survival and proliferation. We observed that the presence of endorepellin in the sponges harboring the colon carcinoma cells caused a
marked suppression of the angiogenic process (Fig. 4e) as compared with the tumor cells themselves (Fig. 4d). Quantification of both sets of experiments using the NIH Image analysis software showed a 74 and 80% inhibition of vessel area around the sponges (p < 0.001) (Fig. 4, f and g, respectively).

To further investigate the role of endorepellin in in vivo angiogenesis, we performed Matrigel plug assays in nu/nu animals (40). To this end, we injected 100 µl of Matrigel supplemented with FGF2 (10 ng/animal) and either BSA or endorepellin (12 µg/animal) into the dorsal subcutaneous regions of ten nu/nu mice. Mice were sacrificed 2 weeks after the injection and the skin removed to analyze the blood vessel formation. Inasmuch as the Matrigel plug is initially avascular, any vessels found within the plug must be, of necessity, new vessels (40). There were striking differences between control and endorepellin-treated samples. In the latter case, there was a marked inhibition of neovascularization around and within the Matrigel plug (Fig. 5b) as compared with the control samples (Fig. 5a). Microscopic examination showed marked ingrowths of new blood vessels in the control samples (Fig. 5c), but essentially little or no blood vessel formation in the presence of endorepellin (Fig. 5d). Quantification of the blood vessel density, as described above, again showed marked (>75%) suppression of new blood vessels in the presence of endorepellin.

Finally, we tested endorepellin during HUVEC tube formation in a collagen matrix, a process thought to mimic morphogenesis (39). The results showed a capillary-like network formation in the control HUVECs (Fig. 5c), which was visible at 4 h, and remained constant for up to 24 h (not shown). In contrast, endorepellin caused a complete block of tube-like formation at concentrations similar to those used in the migration assays (Fig. 5f), whereas no significant effects were obtained with endostatin (Fig. 5g). Interestingly, endostatin was not capable of blocking the activity of endorepellin (Fig. 5h), suggesting that these two proteins possess distinct mechanisms of action (see below).

Collectively, our results indicate that endorepellin is a powerful blocker of angiogenesis and that its effects are long lasting.

**Biological Effects of Endostatin/Endorepellin Interaction**—To further investigate the biological significance of the interaction between endostatin and endorepellin, we performed several VEGF-induced HUVEC migration experiments in which the amount of endorepellin was kept constant while the amount of endostatin was proportionally increased. We chose two concentrations of endorepellin, 1.2 and 3.7 nM (100 and 300 ng ml−1, respectively) that gave suboptimal and optimal inhibition of HUVEC migration. When endostatin and endorepellin were concurrently present, there was an overall inhibition of their activities (Fig. 6, a and b). By plotting the percentage of migrated cells derived from normalized data of five independent experiments, against the increasing molar ratios of endostatin/endorepellin, maximal neutralization was achieved at ~1:1 molar ratio. Thus, the combined effects of endostatin and endorepellin are not additive, but they may lead to an attenuation of their respective anti-angiogenic activities.

**Specific Binding of Endorepellin to Endothelial Cell Surface**—Next, we sought to determine whether endorepellin could specifically bind to the cell surface of HUVECs. We labeled endorepellin with [125I] to high specific activity (~1018 cpm mol−1) and found the predominant 81-kDa band, with a small fraction of labeling going into a 25-kDa fragment (Fig. 7a). To establish the nature of this fragment, we transferred a similar preparation to polyvinylidene difluoride membrane and sequenced the N terminus. This confirmed that the 25-kDa fragment encompassed nearly all the LG3 module, with a specific cleavage between Asn-4196 and Asp-4197 (Fig. 7a). Covalent
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Fig. 3. Recombinant endorepellin inhibits VEGF-mediated chemotactic migration of endothelial cells. a, purification of endorepellin from media conditioned by 293-EBNA cells expressing the 81-kDa endorepellin tagged with His6. Coomassie-stained SDS-PAGE (left) and Western immunoblotting with anti-His6 antibody (right) of negative control media (lanes 1 and 4), flow through (lanes 2 and 5), and 250 mM imidazole eluate (lanes 3 and 6). b, Coomassie-stained SDS-PAGE of purified endorepellin following elution from a DEAE Sephadex chromatography. Lane 1, molecular weight rainbow markers; lane 2, starting material; lane 3, 150 mM NaCl eluate; lane 4, 2 M NaCl eluate. c and d, HUVEC migration assays through fibrillar collagen type I using 10 ng ml⁻¹ VEGF as a chemotactic inducer with or without incubation with various concentrations of endostatin (ES) and endorepellin (ER) as indicated. SFM, serum-free medium. The values represent the mean ± S.E. of quadruplicate determinations. These experiments were repeated three times with comparable results.

High Affinity Binding Sites for Endorepellin on Various Tumor Cell Lines—Next, we wished to test whether endorepellin binding sites could be present on cells other than HUVECs. To this end, we fused the coding region of endorepellin to that of the heat-stable human placental AP (55), a soluble marker that can be readily detected by chemiluminescence’s reagents (42). We isolated several clones that expressed relatively high levels of endorepellin/AP chimeric protein (Fig. 8a) and quantitative analysis, using a standard curve based on AP activity, revealed that 10⁵ clone 6 cells expressed ~4 μg ml⁻¹ 48 h⁻¹. Immunoprecipitation studies using an anti-AP monoclonal antibody linked to agarose showed the predicted sizes of ~141 kDa for the AP and endorepellin/AP chimera, respectively (Fig. 8b). Notably, incubation of endorepellin/AP-conditioned media with HUVEC, MCF7, HT1080, and WiDr cells showed significant binding to the cell surface of all the cells, with the highest binding in WiDr followed by HUVEC, HT1080, and MCF7 (Fig. 8c). No binding was observed with AP alone (not shown), further indicating the specificity of the interaction between soluble endorepellin and the cell surface. We also tested A431 squamous carcinoma cells, but we could not block the endogenous AP even after 1–2 h incubation at 65 °C, indicating that these cells possess an endogenous heat-stable AP. To bypass this point, we performed binding studies of A431 and MCF7 (as a further control) cells using ¹²⁵I-endorepellin as the soluble ligand. The results showed a saturable binding for both A431 and MCF7 cells (Fig. 8, d and f). Scatchard analysis revealed a single receptor population for both A431 and MCF7 cells consisting of ~9 and ~7.5 × 10⁴ sites cell⁻¹, with Kd values of 6.6 and 26 nM, respectively (Fig. 8, c and g). The lower number of receptors on the MCF7 as compared with the HUVECs is in full agreement with the binding studies shown in panel c. Notably, cross-linking experiments of A431 and MCF7 cells also showed the presence of high Mₙ complexes that did not penetrate the gel (not shown) suggesting that similar putative receptors are also present in these two cells.

Endorepellin Has Counter-adhesive Properties for Endothelial Cells—A number of bioactive fragments of extracellular matrix proteins exhibit counter-adhesive activity; that is, they disrupt cell-matrix interactions (52). It has been previously shown that domain V of perlecan, from either mouse or Drosophila, is adhesive for several cell lines when compared with fibronectin, but not for others (36, 49). To address this point, we tested whether endorepellin could mediate HUVEC adhesion. We found a complete lack of HUVEC adhesion to either endorepellin or BSA, in contrast to a robust adhesion to fibronectin or collagen type I (Table I). In competition experiments in which we challenged HUVECs with increasing amounts of endorepellin, we found a progressive inhibition of HUVEC attachment; within minutes the cells rounded up and began to detach in a dose-dependent manner (Fig. 9a). We performed several experiments on fibrillar collagen or plastic and, consistently, endorepellin prevented HUVEC binding to either substratum, with an IC₅₀ of 5–20 nM. In contrast, endostatin did not show any interference with endothelial cell attachment to either fibronectin or collagen I (data not shown).

To verify that the anti-adhesive property of endorepellin was not just limited to endothelial cells, we tested HT1080 fibrosarcoma cells, which do not bind to murine domain V (36), and WiDr colon carcinoma cells (19). In both cases, endorepellin did not support adhesion (Table I). Moreover, specificity of endorepellin counter-adhesive properties was confirmed by the efficient displacement of HT1080 and WiDr attachment to fibronectin with increasing concentrations of recombinant endorepellin, with IC₅₀ of 110 and 40 nM, respectively (Fig. 9b). In contrast, endostatin did not significantly affect the adhesion of either cell line (Fig. 9c). A summary of all the binding data, in which adhesion assays were performed using fibronectin and two concentrations of collagen type I, is provided in Table I. Interestingly, we found that endorepellin not only failed to
support adhesion for HUVECs, but also for most of the tumor cell lines tested, including HeLa, HT1080, WiDr, and M2 tumor cell lines. In contrast, A431 squamous carcinoma cells, which were previously shown to adhere to murine domain V, showed a mean attachment value of 52 \( \pm \) 4\% nearly identical to what has been previously obtained (36). We also found that MCF7 breast carcinoma cells had a similar (50 \( \pm \) 7\%) attachment value. Thus, endorepellin is a powerful anti-adhesive factor for endothelial cells and certain tumor cells, while it is partially adhesive for other tumor cell lines.

**DISCUSSION**

In an *in vivo* screening using the entire C-terminal domain V of human perlecan as bait, we discovered a strong interacting protein comprising the C terminus of human collagen type XVIII, including the anti-angiogenic factor endostatin (44). It has been previously shown, using a cell-free system, that perlecan proteoglycan binds to endostatin, presumably via the heparan sulfate chains (56, 57). We independently confirm these results and further show that a distinct subdomain of perlecan protein core specifically binds to endostatin. Using a battery of deletion mutants, the major binding site was mapped to the second laminin-like G domain of perlecan domain V. Because perlecan and type XVIII collagen/endostatin co-distribute in basement membranes (3, 22, 45, 46, 56), and because endostatin binds *in situ* to vascular basement membranes independently of heparan sulfate (58), we propose that domain V is a binding site for endostatin *in vivo*. Therefore, one outcome of these results, from a physiological point of view, would be that we have discovered an important interaction between the C terminus of perlecan and the C terminus of type XVIII collagen. This interaction could play a key role in the assembly of basement membranes and, perhaps, in the maintenance of their integrity.

Surprisingly, using HUVEC migration assays, we discovered that, while the interaction between endostatin and domain V counteracted their activities, perlecan domain V itself was a powerful anti-angiogenic factor, and hence we named it endorepellin. Endorepellin was active at nanomolar concentrations and was a potent inhibitor of angiogenesis: endothelial cell migration through fibrillar collagen, collagen-induced capillary-like formation, and growth of blood vessels in the CAM and Matrigel plug assays. The action of endorepellin was as strong as endostatin in inhibiting HUVEC migration, and in some experiments was even stronger than endostatin. Interestingly, endorepellin was also capable of counteracting
the angiogenic properties of WiDr colon carcinoma cells in the CAM assays. Notably, these cells synthesize large amounts of perlecan (19), which has been recently shown to bind FGF2 with affinities even higher than the endothelial cell perlecan (59). Thus, it is possible that endorepellin might act in a negative dominant fashion, at least in regard to the inhibition of capillary formation. We recently found that 293-EBNA cells expressing endorepellin do not form tumors in nude mice, in contrast to the wild-type cells, suggesting that endorepellin might also play an anti-tumorigenic role in vivo.

We found a significant number of endorepellin binding sites on HUVECs, with a relatively high affinity constant ($K_d \approx 11$ nM). The specificity of binding was proved by the efficient displacement of the HUVEC-bound $^{125}$I-endorepellin by increasing amounts of cold endorepellin, with an IC$_{50}$ of 27 nM, in good agreement with the affinity constant mentioned above. The presence of putative endorepellin receptor(s) was further corroborated by the presence of high $M_r$ complexes cross-linked to endorepellin. We also found high affinity binding sites on A431 and MCF7 tumor cells using radioligand binding assays similar to those used for HUVECs.

Endorepellin interfered with the adhesive properties of endothelial cells for various substrata, including fibronectin and fibrillar collagen, without directly binding to either protein matrix, and was also anti-adhesive for certain tumor cells derived from colon, neuroectoderm or mesenchyme. This is in agreement with previous studies showing anti-adhesive properties for perlecan in hematopoietic (30), mesangial (31), myoblastic (60), and smooth muscle (61) cells, and a role for perlecan in the suppression of growth and invasion in fibrosarcoma cells (62). However, while endorepellin inhibits tube formation and prevents cell adhesion to fibronectin and other substrata, monomeric endostatin does not. Thus, the two molecules may act via distinct mechanisms. We should point out, however, that oligomeric endostatin and the NC1 domain of collagen type XVIII have been recently shown to effectively inhibit tube morphogenesis (63), indicating that oligomerization is an im-

![Image](Matrigel + FGF2)

![Image](Matrigel + FGF2 + Endorepellin)

![Image](Endorepellin blocks blood vessel ingrowth in the Matrigel plug, and prevents endothelial tube formation induced by fibrillar collagen. a and b, digital images of dorsal skin viewed from the inside, 2 weeks after subcutaneous injection of Matrigel supplemented with FGF2 and either BSA or endorepellin. Notice the decreased neovascularization around the Matrigel plug (asterisk) in the endorepellin-treated samples as compared with the control samples (arrows). Scale bars, 5 mm. c and d, photomicrographs of Matrigel plugs from either control or endorepellin-treated samples, respectively. The ingrowths of new blood vessels are markedly enhanced in the control samples (arrows), as compared with the endorepellin-treated samples. Scale bars = 500 μm. e–h, gallery of light micrographs capturing the production of HUVEC tube-like formation within a collagen type I matrix either alone or following the addition of endorepellin, endostatin, or both. Several concentrations of endorepellin and endostatin (50–150 nM) were tested. In this assay, $4 \times 10^4$ cells were incubated for 24 h, and pictures were taken at various intervals. The images shown are from the 4-h time point. The images at 24 h were identical to those obtained at 12 h (not shown), indicating that the effects of endorepellin are long lasting. These experiments were repeated three times with comparable results. Scale bar, 250 μm.

![Graph](Biological consequences of endostatin/endorepellin interaction. a and b, HUVEC migration assays through fibrillar collagen using 10 ng ml$^{-1}$ VEGF as a chemotactic inducer and preincubation of HUVECs for 30 min with various concentrations of endostatin (ES), endorepellin (ER), or various combinations as indicated. The values are presented as the percentage of maximal migration induced by VEGF, arbitrarily set at 100%. Panel a shows the summary of three independent experiments run in quadruplicate, mean ± S.E. The values in panel b derive from an additional experiment run in quadruplicate, mean ± S.E. SFM, serum-free medium.)
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Fig. 7. High affinity binding sites for endorepellin on endothelial cells. a, autoradiography on a 10% SDS-PAGE of endorepellin (ER) labeled to high specific activity (≈ 10^{12} cpm mol^{-1}) using Iodogen (Pierce). The autoradiograph was purposefully overexposed to show the minor contaminant band of ≈25 kDa. About 15 pmol of the purified 25-kDa band were electroblotted onto a Problott-polyvinylidene difluoride membrane and microsequenced. The first N-terminal amino acid residue is Asp-4197, near the beginning of the LG3 module. The seven amino acid residues match perfectly to the human sequence of perlecain (7, 8). b, covalent affinity cross-linking. HUVECs were incubated with various concentrations of 125I-endorepellin for 2 h as indicated in the bottom, and then incubated for 30 min with 2 mM BS3, a membrane-impermeable cross-linker. The reaction was terminated with 1 M Tris, pH 7.5, and the cross-linked material was separated on 7.5% SDS-PAGE, and visualized by autoradiography. Notice that endorepellin, but not the LG3 module, is complexed with high molecular weight material that does not penetrate the separating gel. c, saturation binding of 125I-endorepellin on HUVECs. Confluent HUVECs in 24-well plates were incubated with increasing concentrations of 125I-endorepellin for 2.5 h at 4 °C in M199 containing 0.1% BSA, washed several times, and extracted in the presence of protease inhibitors (41). Values represent the mean ± S.E. of three independent experiments run in triplicate. Non-specific binding was subtracted from the observed values. d, Scatchard analysis of the data presented in c. Estimates of receptor affinity and total binding capacity were made with the Wizard program in the Sigma Plot 5.0 software package. These experiments were repeated three times with similar results. e, displacement of HUVEC bound 125I-endorepellin by increasing amounts of cold endorepellin. The data represent the mean ± S.E. of two independent experiments run in triplicate. In these experiments, confluent HUVECs (≈10^5 cells/dish) were incubated with 125I-endorepellin (5 nM) plus increasing concentrations of recombinant unlabeled endorepellin, as indicated. The cells were incubated at 4 °C for 3 h, washed three times, extracted, and counted in total.

Fig. 8. High affinity binding sites for endorepellin on various tumor cell lines. a, generation of a cellular system secreting either AP or endorepellin/AP chimeric protein. Lanes 1 and 2 represent conditioned media from either untransfected or AP-transfected 293-EBNA cells. Lanes 3–13 represent media from positive and negative clones of 293-EBNA cells stably transfected with the endorepellin/AP construct. Conditioned media were incubated at 65 °C for 30 min to inactivate endogenous phosphatases, cooled on ice, and then mixed with CospD substrate/chemiluminescent enhancer for 10 min (Great EscAPE™ SEAP system, Clontech), followed by exposure to x-ray film for 5–10 s. One of the strongest clone, (clone 6, lane 4) was amplified and used in the subsequent analyses in b and c. b, identification of AP alone or the chimeric endorepellin/AP protein following immunoprecipitation with a mouse monoclonal antibody (Clone 8B6, Sigma) against human placental AP linked to agarose beads. Coomassie Blue-stained 10% SDS-PAGE of 0.5 ml of conditioned media from control (lane 2), AP-secreting (lane 3), or endorepellin/AP-secreting (lane 4) 293-EBNA cells following incubation with 4 μl of antibody-agarose resin. Because 1 ml of settled resin binds at least 0.5 mg of human placental AP protein, we estimate that 10^5 clone 6 cells express ≈4 μg ml^{-1} 48 h^{-1}. Molecular weight markers are in lane 1. c, binding of endorepellin/AP chimeric protein to various cells. Binding studies were performed using various cell lines (as indicated) incubated with 0.5 ml of serum-free media conditioned by expressing or control 293-EBNA cells for 48 h, using various dilutions as indicated in the left margin. After a 1.5-h incubation at 25 °C, the cells were washed six times, lysed in 1% Triton X-100, 20 mM Tris-HCl, pH 7.5, and processed for AP assays as above. d and f, saturation binding curves of 125I-endorepellin on A431 squamous carcinoma (d) and MCF7 breast carcinoma (f) cells. Confluent cells in 24-well plates were incubated with increasing concentrations of 125I-endorepellin for 2.5 h at 4 °C in M199 containing 0.1% BSA, washed several times and extracted in the presence of protease inhibitors (41). Values represent the mean ± S.E. of two independent experiments both run in quadruplicate. Non-specific binding was subtracted from the observed values. e and g, Scatchard analyses of the data presented in d and f, respectively. Estimates of receptor affinity and total binding capacity were made with the Wizard program in the Sigma Plot 5.0 software package.
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The values are derived from adhesion assays to fibronectin, collagen type I, or endorepellin at the indicated concentrations, mean ± S.E. (n = 4). The numbers are related to the attachment to fibronectin arbitrarily set at 100%; that is, the OD values at 600 nm achieved in the plateau region of the curve, and those of collagen and endorepellin as percentage of the fibronectin plateau. About 80% of the total cells were attached in the plateau region of the fibronectin curve. In these experiments, $5 \times 10^4$ cells were seeded onto 96-well plates previously coated for 18 h at 4°C with the various proteins at the indicated concentrations. After a 1-h incubation, the wells were extensively washed, and the number of adhered cells was estimated by a colorimetric assay.

| Cell type                  | [Fibronectin] 50 nM | [Collagen I] 660 nM | [Collagen I] 50 nM | [Endorepellin] 50 nM |
|----------------------------|---------------------|--------------------|--------------------|---------------------|
| HUVEC, endothelial         | 100 (±5)            | 93 (±2)            | 104 (±5)           | <5                  |
| A431, squamous carcinoma   | 100 (±6)            | 84 (±12)           | 72 (±6)            | 52 (±4)             |
| HeLa, squamous carcinoma   | 100 (±10)           | 92 (±9)            | ND                 | <5                  |
| HT1080, fibrosarcoma       | 100 (±2)            | 100 (±10)          | ND                 | <5                  |
| WiDr, colon carcinoma      | 100 (±6)            | ND                 | ND                 | <5                  |
| MCF7, breast carcinoma     | 100 (±2)            | 182 (±2)           | 169 (±5)           | 50 (±7)             |
| M2, mouse melanoma         | 100 (±5)            | 81 (±3)            | 77 (±3)            | <5                  |

* ND, not determined.

FIG. 9. Endorepellin is counter-adhesive for endothelial, fibrosarcoma and colon carcinoma cells. a, gallery of light micrographs of crystal violet-stained HUVECs adhered to fibronectin following incubation with endorepellin at the indicated concentrations. Briefly, the cells were trypsinized and plated onto fibronectin precoated (50 nM) wells in the presence of increasing concentrations of endorepellin or in the presence of phosphate-buffered saline (control). The cells were then incubated for 1 h, washed, and stained with crystal violet. After washing again, the cells were solubilized with Triton X-100, and the OD at 600 nm was determined. The adhesion assays were conducted in serum-free M199 medium. Scale bar, 100 μm. b and c, displacement of HT1080 (♦) and WiDr (○) cells from fibronectin-coated wells with increasing concentrations of either endorepellin or endostatin, respectively. The calculated IC₅₀ values for HT1080 and WiDr were 110 and 40 nM, respectively. The values represent the mean ± S.E. (n = 4).

migration by endorepellin was neutralized by endostatin. Presumably, this occurs by the tight binding of endostatin to endorepellin that would alter the ability of endorepellin to interact with the cell surface. A logical extension of this hypothesis would be that this binding would also block the other activity (tube formation) of endorepellin. However, we observed that endostatin did not neutralize this activity. Migration of endothelial cells and tube-like formation are two different mechanisms that involve activation of different pathways (33, 65). The former occurs immediately after an angiogenic stimulus has taken place, whereas the latter involves the differentiation of the endothelial cells at the end of the angiogenic response. Thus, it is possible that the two proteins act on different receptors and that they activate similar or overlapping pathways during cell migration, but differ in the morphogenetic process of tube-like formation within a collagen matrix. It is also possible that endorepellin may bind to more than one receptor, each one involved in controlling different cellular mechanisms.

Powerful angiogenesis inhibitors are proteolytically processed forms of basement membrane collagens types IV, XV, and XVIII, the latter two being chondroitin and heparan sulfate proteoglycans, respectively (66). Moreover, proteolytic remodeling of the extracellular matrix can expose cryptic sites within collagen type IV that are required for angiogenesis in vivo (67). Thus, it is likely that perlecan might undergo a similar proteolytic processing in vivo, thereby liberating endorepellin through an endogenous processing mechanism common to most LG domains of laminin (43, 68, 69). The modular nature of perlecan protein core is particularly well suited for selective proteolysis (17, 66) and subsequent release of peptides with biological activity. There are several lines of evidence that support this scenario. First, in our 293-EBNA cells we detected a natural 25-kDa proteolytic cleavage product of endorepellin, which bound to the Ni-NTA column and was also reactive with the anti-His6 antibody, indicating that it represented LG3. This was further confirmed by N-terminal sequencing analysis, which perfectly matched the seven amino acid residues starting with Asp-4197. Second, a similar band was previously shown to represent a proteolytic fragment of murine domain V generated by cleavage just before the beginning of LG3 (36, 37). This protease-sensitive region, which starts with the sequence DAPGQYG, is completely conserved between mouse (6) and human (7, 8), thus demonstrating that a specific cleavage of an Asn–Asp bond (at positions 3514–3515 and 4196–4197, for the mouse and human counterpart, respectively) had occurred near the N terminus of LG3. Mutational analysis indicated that Asp, but not Asn, is crucial for processing of mouse endorepellin (37), possibly by a specific, yet to be discovered, Asp-N endoproteinase. In our study the LG3 module failed to be cross-
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linked to surface proteins, suggesting that this part of endorepellin is dispensable for surface binding. Third, an identical proteolytic fragment of ~25 kDa, cleaved at the same position as the mouse, was detected in the urine of patients with end-stage renal failure (70). This indicates that the LG3 module is present in the human serum at relatively high concentrations, since this LG3 was found at concentrations of ~10 mg liter⁻¹ of urine (70). Fourth, we have recently discovered an additional proteolytic cleavage site (between Gly-3774 and Asp-3775) within the LG1 subdomain that leads to the release of almost the entire endorepellin lacking only the first 88 amino acid residues.³ While this has not been proven to occur in tissues, it is plausible to take place because of the specificity of the cleavage site and the relatively high amounts of this fragment that we obtained after purification in which the mixture of protease inhibitors was suboptimal. Circulating forms of endorepellin may be involved in the homeostatic control of angiogenesis as previously proposed for endostatin, whose levels can reach 0.3 mg liter⁻¹ of blood (56). We would like to put forward a provocative hypothesis, that is, it has been nearly three decades since its discovery that extracts of cartilage contain potent anti-angiogenic factors (71, 72), and because perlecan is highly expressed in cartilage, both during development and adulthood (9, 29, 73), endorepellin could conceivably be generated from the active remodeling of cartilage that occurs during normal aging, inflammation or any other condition that leads to cartilage turnover.

We do not yet know the precise mechanism of action of endorepellin. Two cell-surface proteins might be involved, either separately or in conjunction, namely, β1 integrin and α-dystroglycan, both of which have been shown to interact with perlecan domain V (36, 75, 76). In the case of α-dystroglycan, perlecan domain V was the strongest ligand (Kₐ of 3 nM) and required LG1 and LG2 modules, whereas LG3 module by itself had much lower affinity (76). In agreement with these in vitro binding assays, perlecan and α-dystroglycan co-localize at the neuromuscular junctions (77, 78) where they may serve as cell-surface acceptors for acetylcholinesterase. Interaction between perlecan and α-dystroglycan, together with laminin, may also play a key role in the assembly of basement membranes during early development (74). Experiments are underway to address these important issues.

Recent experimental tests on tumor-bearing animals are encouraging because protein-based inhibitors, such as endostatin, have the following three major advantages. 1) They can reduce the tumors to a manageable size. 2) They do not induce resistance, and 3) their toxicity is low (66). Endorepellin is a novel natural inhibitor of angiogenesis, and its use in cancer therapy has additional advantages insofar as endorepellin may also exert an anti-adhesive action on certain tumor cells. Thus, we predict that if these protein-based agents are used in concert with traditional therapies, which target neoplastic cells directly, we may manage, or even cure, some forms of cancers that are currently incurable.

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