Laminin Modulates Morphogenic Properties of the Collagen XVIII Endostatin Domain*

Kashi Javaherian‡‡, Susan Y. Park‡, Winfried F. Pickl†, Kenneth R. LaMontagne‡‡, Robert Tjin Tham Sjin‡, Stephen Gillies**, and Kin-Ming Lo**

From the ‡Department of Surgery, Children’s Hospital, Harvard Medical School, Boston, Massachusetts 02115, **Lexigen Pharmaceuticals Corporation, Lexington, Massachusetts 02421, and the ¶Institute for Immunology, University of Vienna Medical School, A-1090 Vienna, Austria

We have shown previously that the oligomeric endostatin domain of collagen XVIII (NC1) functioned as a motility-inducing factor regulating the extracellular matrix-dependent morphogenesis of endothelial cells. This motogenic activity gave rise to structures resembling filopodia and lamellipodia and was dependent on Rac, Cdc42, and mitogen-activated protein kinase. Here, we demonstrate that these properties of endostatin are primarily mediated by laminin in the basement membrane and heparan sulfates on the cell surface. The sites of interaction between laminin and oligomeric endostatin include the N-terminal regions of all three laminin chains (amino acids 204–1243 of the α chain, 932–1161 of the β chain, and 150–965 of the γ chain). A monoclonal antibody that blocks the interactions between endostatin and laminin was utilized to inhibit the motogenic activity of endostatin. In parallel, we have engineered selective point mutations and produced recombinant forms that lack binding to heparan sulfates on the cell surface. Our data are consistent with a model of endostatin with two binding sites: one mainly to laminin in the basement membrane and the other to heparan sulfates on the cell surface. The two binding domains on endostatin appear to be separate with the possibility of some overlap between the two sites.

Endostatin, a 183-amino acid proteolytic cleavage fragment corresponding to the C terminus of collagen 18, has been the subject of investigation by a number of laboratories because of its anti-tumor activity and its potential application as an anti-angiogenic cancer therapeutic (1–3). Previously, endostatin has demonstrated anti-angiogenic properties in vivo and in vitro (4, 5). Although a number of physical and biochemical properties have been described, the mechanism of endostatin function remains unknown. The crystal structure of both murine and human endostatin have been elucidated and show a noncovalently held dimer at high concentration required for crystallization (6, 7). The presence of two internal disulfide bonds results in a highly folded and compact structure. Endostatin monomer binds one atom of zinc via noncontiguous amino acid residues (7). The functional importance of zinc binding is yet to be determined. The heparin binding property of endostatin, by which it was originally isolated, is mediated by arginine-rich clusters spread over the three-dimensional globular surface of the protein (6, 8).

Understanding the molecular basis of endostatin activity may elucidate the specific biochemical properties that enable its anti-angiogenic function and suppression of tumor growth. Recently, we have demonstrated that the carboxyl end of collagen 18, which features an oligomeric structure, plays an essential role in endothelial migration and morphogenesis in a HUVEC1 tube formation assay on Matrigel (9). This phenomenon could not be demonstrated with the endostatin monomer. Furthermore, an analogous region from collagen 15 failed to induce similar morphological changes. In a parallel paper, it was shown that the Caenorhabditis elegans collagen 18 homologue, cle-1, promotes neuronal motility through its trimerization-competent NC1 domain (10). Recently, data were presented suggesting that endostatin binds to integrins (11) and tropomyosin (12). A more detailed investigation by a different group implicated a cell surface glypicin as a low affinity surface receptor for endostatin (13) with the binding strictly mediated by a heparan sulfate-GAG region of glypican.

Morphogenesis of capillary-like tubes by placement of HUVECs on a basement membrane substratum recapitulates the structural features of vessel formation. The tube assay provides a system to investigate protein-protein interactions between the cell surface and the extracellular matrix. Here, we report that the motogenic properties of oligomeric endostatin in the Matrigel tube assay are mediated via two paths: first by its binding to laminin in the basement membrane and additionally by its association with heparan sulfate on the cell surface. These binding sites on endostatin are distinct, although there may be some overlap between the two domains. Based on our data we conclude that the oligomeric endostatin domain of collagen 18 is capable of binding laminin and a cell surface heparan sulfate with the possibility of providing a bridge between the two of them.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins—Expression and purification of recombinant fusion proteins in mammalian cells including human Fc-endostatin dimer and human NC1-His, of c18 have been described previously (9, 14). For the expression of Fc-endostatin (R27A, R139A), the mutations were introduced to pdcs-Fe(D4K)-

* This work was supported by Entremed Corporation and by grants from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed: Children’s Hospital Boston, Enders 470, 320 Longwood Ave., Boston, MA 02115. Tel.: 617-355-4485; Fax: 617-566-6467; E-mail: kashi.javaherian@chb.harvard.edu.

† Present address: Novartis Pharmaceuticals, 556 Morris Ave., Summit, NJ 07901-1398.

Snow and by guest on July 26, 2018http://www.jbc.org/ Downloaded from
endostatin by the overlapping polymerase chain reaction technique (18), using the primer 5'-EATC GCC GCC CCC GGC GAT GGC TTC CGA and antisense primer 5'-AA GTC GCC CCC GGC GAT GCC CC to introduce the Arg95 to Ala substitution (in bold type) and sense primer 5'-TAC TGT GAG ACG TGG GCT GAC GAG GC and antisense primer 5'-CTC AGG CCA CGT AGC ACA to introduce the Arg129 to Ala substitution (in bold type). The mRNA extracted from these hybridoma-conditioned media were affinity-purified on protein A-Sepharose (Repligen Corp. Needham, MA). Recovery of bound protein was achieved by elution with 0.1 M citrate, pH 3, followed by neutralization using 2 M Tris, pH 8. Generation of the endostatin fragment from the Fe fusion proteins was achieved by digestion with enterokinase, which recognizes the D4K sequence engineered between the ES and receptor segments. Further purification was achieved by size exclusion chromatography on Sephacryl-200 (Pharmacia Corp.).

ELISA and Immunoprecipitation—Hybridoma-conditioned media were screened by ELISA based on affinity to 200 ng of human endostatin in 0.1 M sodium bicarbonate, pH 9.6, bound to solid polyvinylcarbonyl substrate (Falcon). Nonspecific binding was blocked with 2% (w/v) BSA (Sigma) in Dulbecco's phosphate-buffered saline (Sigma). Affinity complexes were measured by the addition of secondary antibody goat anti-mouse IgG-horseradish peroxidase, followed by colorimetric detection using 2.2'-azino-di-3-ethyl-benzthiazoline-6-sulfonate (substrate) (Kirkgaard Perry Laboratories). Absorbance was measured on a SpectraMax (Molecular Devices) spectrophotometer at 405 nm. Hybridoma-conditioned media were screened for binding to endostatin by ELISA. Binding reagents (final concentration, 10 μg/ml) were incubated in wash buffer containing 2500 units/ml heparin (Baxter Hyland Immuno, Vienna, Austria) at a cell density of 1 × 10^5 cells/ml on ice for 15 min. The binding reagents (antibodies) were preincubated in wash buffer alone or in wash buffer containing 2500 units/ml heparin (Baxter Hyland Immuno, Vienna, Austria) at a cell density of 1 × 10^5 cells/ml on ice for 15 min. Subsequently, cells (5 × 10^5) were washed twice with wash buffer and binding of primary reagents was detected by goat anti-human Ig conjugated with Alexa 488 or with goat-anti mouse Ig conjugated with Oregon Green (Molecular Probes; final concentration, 20 μg/ml). After 30 min of incubation on ice and two washes with wash buffer, the cells were analyzed by flow cytometry on a FACScan (BD Scalcium). Beckton Dickinson).

RESULTS

Preparation of Oligomeric and Monomeric Forms of the Collagen XVIII Endostatin Domain—For our study, endostatin dimer was generated by two methods. First, fusion of endostatin to the Fe domain of human immunoglobulin γ1 results in proximal dimerization by way of discrete monomer linkage to the disulfide bonded Fe fragment. Second, based on crystal structure evidence showing dimer association at glutamine 7, a mutation to cysteine yielded a covalently linked endostatin dimer conjugated to Fc (9). Upon enterokinase digestion, endostatin dimer was released. Further purification on S-200 gel filtration column resulted in an artificial endostatin dimer where the two endostatin monomers are covalently linked by a disulfide bond at amino acid position 7 (7, 9). Both types of endostatin dimer (Fc-endostatin and disulfide-bonded (Q7C) endostatin), plus trimer (NC1) (a 38-kDa degradation product at the C terminus of collagen 18 that includes the endostatin region) demonstrated properties distinct from endostatin monomer.

Extracellular Matrix Conditioned with Collagen XVIII NC1 Is Sufficient to Promote Motogenic Activity—While examining the temporal requirements for ES domain oligomers to induce motogenic activity, we noted that extracellular matrix could be conditioned with c18 NC1 (ES trimer) or ES dimers, followed by change into medium without ES derivatives. Subsequent addition of cells resulted in full inhibitory activity. As demonstrated previously (9), collagen 18 NC1 and ES dimers conditioned the Matrigel to promote morphological changes of the cells in the tube assay, whereas endostatin monomer lacked such an activity (Fig. 1). These data suggested the presence of endostatin-binding proteins in extracellular matrix preparations such as Matrigel.

Identification of Laminin as a Predominant Endostatin-Binding Protein in Extracellular Extracts—To identify proteins interacting with the ES domain during in vitro endothelial tube formation, we utilized the Fe-endostatin fusion protein, which retains motogenic activity by virtue of ES dimerization (9) but also allows direct immunoprecipitation of ES-associated proteins by interaction of the Fe region with protein A-agarose. Upon incubation of Matrigel with Fe-endostatin and subsequent addition of protein A-agarose, two high molecular mass bands above 200 kDa were co-precipitated. No such protein bands were co-precipitated by parallel incubation with Fe alone (Fig. 2A). The presence of excess endostatin dimer in the immunoprecipitation solution completely blocked binding of Fe-endostatin to Matrigel-derived laminin (data not shown). In
situ trypsin digestion of these protein bands followed by mass spectrum fingerprinting resulted in the identification of these protein bands as different chains of murine laminin-1 (data not shown).

In some immunoprecipitation experiments, another minor protein band migrating at 150 kDa was detected. Amino acid sequencing of this protein established its identity as entactin, which is known to be tightly associated with laminin. The above data do not rule out the possibility that other proteins constituting the basement membrane also bind endostatin but were not detected because of a much lower concentration, relative to laminin. In addition, high molecular mass proteins of the basement membrane (subunit molecular mass > 400 kDa) may have also escaped our detection because of the constraints of gel electrophoresis size separation.

In a separate experiment, anti-laminin polyclonal antibodies were employed to immunoprecipitate endostatin monomer, dimer, or NC1 bound to purified laminin-1 (Fig. 2B). Bindings were observed mainly to dimer and NC1 with little affinity for monomer, consistent with our previous data.

Laminin-Endostatin Binding Is Enhanced by Endostatin Oligomerization—Timpl and co-workers (2) have reported that the binding affinity of laminin to NC1 is 100-fold higher than to monomer. Laminin complexes strongly with NC1, endostatin dimer, Fc-endostatin, but not endostatin monomer (Fig. 3A). Binding measurements were carried out by employing a series of endostatin concentrations, with laminin serving as the ligand (Fig. 3B). Our estimates of half-maximal binding concentrations are \(7 \times 10^{-9}\) and \(4 \times 10^{-9}\) M for endostatin dimer and Fc-endostatin, respectively. A very weak binding for endostatin monomer was observed (data not shown).

Abrogation of Motogenic Properties of ES Domain Oligomers by Exogenous Laminin—Purified murine laminin-1 was directly introduced at the time of cell seeding on Matrigel, in combination with endostatin dimer or alone. A series of concentrations of laminin were employed (Fig. 4A). The exogenous laminin completely blocks the effect of dimer in disrupting tube formation. At a concentration of \(-40\) \(\mu\)g/ml of laminin, the formation of tubes were almost uninhibited. Thus a stoichiometric ratio of 1:1 (laminin molecular mass = 800,000 Da; endostatin dimer molecular mass = 40,000 Da) is sufficient to limit dimer activity. A similar experiment employing perlecan (a gift from Dr. John Whitelock) instead of laminin failed to block the effects of dimer (data not shown). Exogenous laminin was capable of blocking the effects of NC1 similar to the dimer (data not shown).

Matrigel is rich with laminin. The fact that exogenous laminin can block motogenic properties of endostatin dimer raises questions. A possible explanation is that interactions of the cells with solidified Matrigel (required for formation of tubes) is a slow process (hours) because of inefficient kinetics, whereas the binding of exogenous laminin solution to the cells is a very fast reaction.

Protease Digestion of Laminin Results in Identification of Regions Binding to Endostatin—To map the domain of laminin involved in binding to endostatin, laminin was digested with Staph A (strain V8), which cleaves peptide bonds on the carboxyl side of glutamic and aspartic acids. Digestion was followed by immunoprecipitation using Fc-endostatin. The protein complexes were subjected to SDS-PAGE under reducing and nonreducing conditions (Fig. 5A, lanes 4 and 5). The non-

**Fig. 1.** Tube formation of endostatin as a function of its oligomeric state. Assays were performed as described under “Experimental Procedures.” The concentrations were: monomer, 3000 nM; dimer, 50 nM; and NC1, 50 nM.

**Fig. 2.** A, immunoprecipitation of Matrigel by Fc-endostatin. 150 \(\mu\)l of Matrigel was diluted up to 10 ml in 1% Nonidet P-40, 50 mM Tris, 0.15 M NaCl, pH 7.5, and used in each immunoprecipitation reaction. hFc-endostatin and hFc were bound to protein A (\(\sim 100 \mu\)l) and incubated for 16 h at 4°C. The sample was washed twice in the above buffer and twice in 0.1% Nonidet P-40 with the same buffer. The proteins were extracted in reducing and denaturing sample buffer and analyzed by SDS-PAGE (4–20% gradient gel; Bio-Rad) followed by staining with Coomassie. Lane 1, molecular mass markers; lane 2, 10 \(\mu\)g of Matrigel; lane 3, 20 \(\mu\)g of purified laminin-1; lane 4, 20 \(\mu\)g of hFc; lane 5, 30 \(\mu\)g of hFc-endostatin; lane 6, hFc (control) immunoprecipitation; lane 7, Fc-endostatin immunoprecipitation of Matrigel resulted in co-immunoprecipitation of laminin (Note: in Fc-endostatin, endostatin comprises 40% of total molecular mass). B, anti-laminin antibodies immunoprecipitate oligomeric endostatin. 2 \(\mu\)l of anti-laminin polyclonal antibodies (Sigma) and 10 \(\mu\)g of laminin were used to immunoprecipitate endostatin for each reaction. The Western blot membrane was probed with polyclonal antibodies to laminin and endostatin. Under reducing conditions both endostatin monomer and dimer migrate to the same position. The secondary antibody reacts with IgG. Lanes 1–3 are IP products. Lane 1, 6 \(\mu\)g of monomer; lane 2, 6 \(\mu\)g of dimer; lane 3, 10 \(\mu\)g of NC1.
reduced protein fragment (indicated by an arrow) was analyzed by trypsin digestion followed by mass spectroscopy. The data are presented in Table I. In general, a large number of peptides were identified between the two indicated terminals of each chain. Based on these data, given the absence of any peptides from regions I and II, we conclude that the N-terminal regions of the three chains participate in the complex formation with endostatin; from the α chain amino acids 204–1243, from the β chain amino acids 932–1161, and from the γ chain amino acids 150–965 comprise the laminin fragment coming in contact with endostatin. The proximity of the disulfide-bonded hinge region between regions II and III raises the possibility that it may be present in the isolated laminin fragment. Inclusion of the hinge region could account for the identification of interacting regions from all three chains, although in actuality endostatin may be in contact with one or two of the chains (Fig. 5B).

Extensive trypsin digestion of laminin did not result in identification of a smaller fragment (data not shown). It is of some interest to point out that the mapped β region is much shorter than the other two chains.

**Laminin-binding and Heparin-binding Surfaces of Endostatin**—The heparin binding properties of monomeric endostatin are well established (8). Based on our preliminary experiments and those reported by others, a construct of human Fc-endostatin was engineered such that two arginines, 27 and 139, were mutated to alanine (6, 7). It has been reported previously that this mutant lacks heparin binding (8). Analysis of the binding affinity of different endostatin constructs to BSA-heparin (Sigma) was undertaken. A general ELISA profile is shown in Fig. 6A. Both endostatin monomer and dimer bind heparin, although dimer shows a slightly higher affinity for heparin. The Fc-endostatin mutant (R27A/R139A) fails to bind to heparin as expected.

To obtain a more precise picture, we carried out binding measurements of different forms of endostatin to heparin. The data are presented in Fig. 6B. Both proteins Fc and Fc-endostatin (R27A/R139A) did not demonstrate binding to heparin. The half-maximal binding concentration for human endostatin monomer to heparin is 3.8 × 10⁻⁶ M, that for human Fc-endostatin 1.7 × 10⁻⁷ M, and that for Fc-endostatin (Q7C) was found to be 3.6 × 10⁻⁷ M. A similar binding profile was observed for NC1 (data not shown). Surprisingly, a higher affinity (1.6 × 10⁻⁸ M) was observed with endostatin dimer (Q7C) upon removal of Fc.

The Fc-endostatin mutant lacking heparin affinity failed to cause substantial morphological changes attributed to dimer (Fig. 6C). These results clearly demonstrate that the heparin-binding domain of endostatin dimer is important for conferring morphological changes in the tube assay.

Based on solid phase binding studies, Fc-endostatin (R27A/R139A), which lacks binding to heparin, demonstrates strong binding to purified laminin (Fig. 3A). Similar binding profiles were obtained for control endostatin dimers and Fc-endostatin mutant (Fig. 3B). However, co-immunoprecipitation of endostatin mutant (R27A/R139A) demonstrated considerably less affinity to either Matrigel-derived laminin or purified laminin (Fig. 6D) using a series of laminin concentrations. In contrast to wild type endostatin, which bound laminin strongly, heparin binding deficient endostatin demonstrated a much lower affinity for laminin. The situation is to some extent similar to a large number of monoclonal antibodies showing strong binding to their ligands in solid phase format but lacking high affinity.
in immunoprecipitation assays. One such example was encountered in this study with a monoclonal antibody directed against a linear peptide fragment of endostatin (called PDM; discussed later). It strongly binds endostatin in an ELISA but is not capable of immunoprecipitating endostatin. Therefore, a likely explanation is that removal of two arginines important for heparin binding has caused a change in the conformation of endostatin, lowering its affinity for laminin. Solid phase assays are not very sensitive to such structural changes because by immobilizing a ligand on a solid surface, linear protein sequences are exposed, which are otherwise parts of three-dimensional structures of the native molecule.

Abrogation of Endostatin-Laminin but Not Endostatin-Heparin Association by Neutralizing Monoclonal Antibody 12C1

A number of monoclonal antibodies were prepared against human endostatin monomer. These were screened for inhibiting the scattering activity of dimer in tube assay. 12C1 was a highly potent neutralizing monoclonal that showed more than 90% inhibition of dimer activity at a molar ratio of 1:2 (monoclonal:dimer) (Fig. 7A). In parallel, another monoclonal was generated against a linear peptide of endostatin, called PDM (see “Experimental Procedures”). A set of overlapping peptides corresponding to the complete sequence of endostatin were employed to map the epitopes of 12C1, using PDM as a control (see “Experimental Procedures”) using ELISA experiments. In contrast to PDM, which bound to its corresponding peptide, 12C1 failed to show any binding to linear peptides derived from endostatin (data not shown). PDM failed to inhibit scattering activity of endostatin dimer (Fig. 7A).

By ELISA, monoclonal 12C1 blocks binding of endostatin dimer to laminin, whereas PDM had no effect (Fig. 3A). It also blocks binding of purified laminin and Matrigel to endostatin dimer in the immunoprecipitation experiments (Fig. 7B). In the same context, 12C1 immunoprecipitated both endostatin monomer and R27A/R139A monomer (Fig. 7B). We conclude that monoclonal 12C1, by blocking binding of oligomeric endostatin to laminin, prevents scattering morphology associated with oligomeric endostatin.

Fluorescence-activated Cell Sorter Analysis Demonstrates That Heparin Binding of Endostatin Is Directed to Cell Surface—To investigate binding of oligomeric endostatin to cell surface, we employed flow cytometry. At 10 μg/ml, Fc-endostatin bound to HUVECs, causing a shift in the population of cells (Fig. 8). This binding was inhibited by heparin. The mutant (R27A/R139A) did not exhibit binding to the cell surface, consistent with the notion that binding of endostatin to the cell surface is mediated by the heparin-binding region of endostatin. It should be pointed out that the observed changes for endostatin concentration employed is in agreement with its low affinity for heparin, as determined earlier in solid phase assays.

MAPK Activation by Oligomeric Endostatin Is Blocked by Neutralizing Monoclonal 12C1—Previously, we have demonstrated that the scattering properties associated with oligomeric endostatin in the tube formation assay results in MAPK activation inside the cells (9). Endostatin monomer lacks such an activity. The MAPK pathway regulates numerous aspects of cell growth, differentiation, and response to extracellular stimuli.

In view of the observation that neutralizing monoclonal 12C1 blocked endostatin binding to laminin and inhibited induction of cell motility, a MAPK activation assay was undertaken in the presence of monoclonal 12C1 and non-neutralizing monoclonal PDM. The results are shown in Fig. 9. 12C1 blocked MAPK activation, whereas PDM failed to do so. We conclude that laminin together with oligomeric endostatin are required for MAPK activation. Blockade of laminin interaction with endostatin inhibits MAPK signaling.

Interactions of Endostatin Alone with HUVEC Surface Does Not Appear to Be Mediated by Integrins—We employed a series of monoclonal antibodies which are directed to various integrin α chains (α1, α2, α3, α4, αv, and α6) (Chemicon International Inc.). These monoclonals block the functions of their corre-
sponding integrins. Each was tested in the tube assay to find out whether their presence has any effect on tube formation. None of them resulted in any significant morphological changes (data not shown). In another experiment, cells were incubated with each of these monoclonals followed by the addition of dimer. Binding of monoclonals to cell surface (as established by flow cytometry) failed to disrupt scattering properties of endostatin dimer (data not shown).

We are inclined to conclude that the scattering properties of endostatin are not mediated by integrins. However, it is possible that binding of endostatin to basement membrane molecules may modify its interactions with integrins. The consequence of interactions between endostatin-laminin and integrins requires further investigation.

**DISCUSSION**

Laminins are important for cell adhesion, migration, growth, and differentiation (17–19). They are heterotrimeric glycopro-
teins that constitute a major component of basement membranes. The three protein chains incorporated are designated α, β, and γ (20, 21). As a result of chain variation, different types of laminin are formed. The present study has utilized murine laminin-1 (22). It is an important ligand for a number of cell surface integrins (23).

Endostatin has been shown to be associated with the basement membrane (24–26). As a consequence, endostatin is widely distributed in a large number of tissues and is a component of vessel walls and large arteries like the aorta (2, 27).

Based on the tube formation assay and in vitro studies, we have identified laminin as the primary basement membrane protein responsible for binding to endostatin. Endothelial cell tube morphogenesis, as demonstrated in our work, requires the presence of a number of basement membrane proteins. Replacing Matrigel with laminin failed to produce similar tubes (data not shown).

Our data indicate that the effects of oligomeric endostatin in the disruption of tube formation are mediated by laminin in the basement membrane and heparan sulfate on the cell surface. The N-terminal regions of the three chains of laminin are found to participate in the formation of a complex with oligomeric endostatin. Recently, in collaboration with another group of investigators, we have demonstrated that Chinese hamster ovary cells deficient in heparan sulfate expression fail to show scattering activities associated with endostatin dimer in the tube assay.2

How can we reconcile the above data with those of collagen 18 as a constituent of basement membrane? It is possible that collagen 18 has multiple sites for attachment to the basement membrane, with endostatin domain as a site of interaction with laminin. In that case, one should probably distinguish between the function of endostatin in the context of collagen 18 and its mode of action delivered as an exogenous recombinant protein.

2 F. H. Blackhall, C. L. Merry, A. Henrioud, J. A. Deakin, S. Y. Park, J. Folkman, K. Javaherian, and J. T. Gallagher, unpublished data.
As a part of collagen 18, endostatin may also be associated with proteoglycans in basement membrane, in addition to laminin, although there is evidence indicating that endostatin binding to the subendothelial matrix of vasculature is independent of heparan sulfate (26). Under certain circumstances, the endostatin as a part of collagen may bind cell surface GAG proteins and affect the structure of blood vessel formation.

Does a cell surface receptor exist for endostatin? We do not know the answer to this question. A recent report implicates glypic-1 as a low affinity receptor for endostatin (13). Heparan sulfate proteoglycans on the cell surface play an important role in biological functions (28). Because binding to glypican-1 has been shown to be completely mediated by heparan sulfate domain, it is important to identify unique sequences in glypican that are not shared by other GAG proteins. The authors also report a dissociation constant in the vicinity of $10^{-11}$ M for endostatin affinity to glypican. Our cell surface binding studies of endostatin show a much lower affinity, consistent with a half-maximal binding concentration of $10^{-8}$ to $10^{-7}$ M observed for endostatin binding to heparin.

Both laminin and collagens are known to bind to integrins on the cell surface (19). Our results do not rule out the possibility that endostatin may directly or indirectly (i.e. via laminin) bind to integrins. However, our attempts at several of the monoclonal antibodies directed to integrins in the context of tube formation assay have failed to identify an integrin mediating the action of oligomeric endostatin. An earlier report by a group of investigators demonstrated an interaction between endostatin and integrins (11).

Deletion of the NC1 domain of the C. elegans collagen 18 homologue, cle-1, has been shown to result in viable fertile animals that display multiple cell migration and axon guidance defects (10). In a similar manner, the chemotactic netrin plays an important role in regulation of branching and extension of motor neuron axons from the ventral nerve cord (29). Furthermore, in a separate investigation employing Xenopus laevis, it has been demonstrated that laminin-1 converts netrin-mediated attraction of axons in the developing optic pathway into repulsion (30).

In conclusion, we propose a model in which recombinant oligomeric endostatin is capable of binding to both laminin-1 in the basement membrane and cell surface heparan sulfates. This raises the possibility that the endostatin domain of collagen 18 may provide a bridge between basement membrane and the cell surface. In that case, the delivered exogenous recombinant endostatin may serve as a dominant negative. It remains to be seen how these properties of endostatin affect capillary tube morphogenesis, endothelial migration, and its antitumor activity.

Acknowledgments—We are greatly indebted to Prof. Judah Folkman in whose laboratory this work was carried out. His support and encouragement were essential for undertaking this investigation. We are also grateful to Calvin Kuo for many suggestions and advice. Special thanks to Prof. Brian Seed at Harvard Medical School in whose laboratory the fluorescence-activated cell sorter studies were initiated. Correspondence with Prof. Rupert Timpl was helpful in elucidating the laminin regions binding to endostatin. We thank John Lincecum for his help with the immunohistochemical experiments. The technical assistance of Gustave Alberti is appreciated. We are grateful to Brenda Figueroa and Kristin Gullage for help in preparing this manuscript. Protein sequencing was performed at the Beckman Research Institute, City of Hope, funded by National Institutes of Health.

REFERENCES

1. O'Reilly, M. S., Boehm, T., Shing, Y., Fukui, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. H., and Folkman, J. (1997) Cell 88, 227–285.
2. Sasaki, T., Fukui, N., Mann, K., Gohring, W., Olsen, B. H., and Timpl R. (1998) EMBO J. 17, 4249–4256.
3. Dhanabal, H., Ramchandran, R., Volk, R., Stillman, I. E., Lombardo, M., Iruela-Arispe, M. L., Simon, M., and Sukhatme, V. P. (1999) Cancer Res. 59, 189–197.
4. Bergers, G., Javaherian, K., Lo, K.-M., Folkman, J., and Hanahan, D. (1999) Science 284, 808–812.
5. Sim, B. K. L., Fogler, W. E., Zhou, X. H., Liang, H., Madsen, J., Lou, K., O'Reilly, M. S., Tomaszewski, J. E., and Fortier, A. H. (1999) Angiogenesis 3, 41–51.
6. Hohenester, E., Sasaki, T., Olsen, B. H., and Timpl, R. (1998) EMBO J. 17, 1655–1664.
7. Ding, Y.-H., Javaherian, K., Lo, K.-M., Chopra, R., Boehm, T., Lanciotti, J., Harris, R. A., Li, Y., Shapiro, R., Hohenester, E., Timpl, R., Folkman, J., and Timpl, R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10443–10448.
8. Sasaki, T., Larsson, H., Kreuger, J., Salmivirta, M., Claesson-Welsh, L., Lindahl, U., Hohenester, E., and Timpl, R. (1999) EMBO J. 18, 6240–6248.
9. Kuo, C., LaMontagne, K., Garcia-Cardenas, G., Ackley, B., Kalman, D., Park, S., Christofferson, R., Kamihara, J., Ding, Y.-H., Lo, K.-M., Gilles, S., Folkman, J., Mulligan, R., and Javaherian, K. (2001) J. Cell Biol. 152, 1233–1246.
10. Ackley, B. D., Crew, J. R., Elamana, H., Pihlajaniemi, T., Koo, C. J., and Kramer, J. M. (2001) J. Cell Biol. 152, 1219–1232.
11. Rehn, M., Veikola, T., Kukk-Vaaler, E., Nakamura, H., Illmen, M., Lombardo, C. R., Pihlajaniemi, T., Alitalo, K., and Vuori, R. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1024–1029.
12. MacDonald, N. J., Shivers, W. Y., Narum, D. L., Plum, S. M., Wingard, J. N., Fuhrmann, S. R., Liang, H., Holland-Linn, J., Chen, D. H., and Sim, B. K. (2001) J. Biol. Chem. 276, 25190–25196.
13. Karumanchi, S. A., Jha, V., Ramchandran, R., Karihaloo, A., Tsiokas, L., Chan, B., Dhanabal, H., Hanai, J., Venkataraman, G., Shriver, Z., Keiser, N., Kalluri, R., Zeng, H., Mulligan, R., Roop, R. D., Hagiwara, K., Yamaguchi, Y., Sasselekharam, R., Cantley, L., and Sukhatme, V. P. (2001) Mol. Cell 7, 811–822.
14. Li, R.-M., Sufo, Y., Chen, J. Li. Y., Lan, Y., Kong, S. M., Chen, L., An, Q., and Gilles, S. (1998) Protein Eng. 11, 495–500.
15. Daughtery, B. L., DeMartino, J. A., Law, M. F., Kawka, D. W., Singer, I. I., and Martin, G. R. (1997) Cancer Res. 57, 2476–2480.
16. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 310–311, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
17. Grant, D. S., Tashiro, K. I., Segui-Real, B, Yamada, Y., Martin, G. R., and Kleinman, H. K. (1989) Cell 58, 943–943.
18. Ghosh, S., and Stack, M. S. (2000) Microsc. Res. Tech. 51, 238–246.
19. Belkin, A. M., and Stepp, M. A. (2000) Microsc. Res. Tech. 51, 280–301.
20. Colognato, H., and Yurchenco, P. D. (2000) Dev. Dynamics 218, 213–234.
21. Tunggal, P., Smyth, N., Paulusson, M., and Ott, M. C. (2000) Microsc. Res. Tech. 51, 214–227.
22. Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J. M, and Martin, G. R. (1979) J. Biol. Chem. 254, 9933–9937.
23. Hynes, R. (1992) Cell 69, 11–25.
24. Halfter, W., Dong, S., Schurer, B., and Cole, G. J. (1998) J. Biol. Chem. 273, 25404–25412.
25. Saarela, J., Rehn, M., Oikarinen, A., Autio-Harmainen, H., and Pihlajaniemi, T. (1998) Am. J. Pathol. 153, 611–626.
26. Chang, Z., Choon, A., and Friedl, A. (1999) Am. J. Pathol. 155, 71–74.
27. Misse, N., Sasaki, T., and Timpl, R. (1999) FASEB J. 13, 1743–1750.
28. Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lancecum, J., and Zako M. (1999) Annu. Rev. Biochem. 68, 729–777.
29. Lim, Y.-S., Mallapur, S., Kao, G., Ren, X.-C., and Wadsworth, W. G. (1999) J. Neurosci. 19, 7045–7056.
30. Hynes, R. E., Shaw, D., Tessler-Lavigne, M., Poo, M.-M., and Holt, C. (1999) Nature 401, 69–73.
Laminin Modulates Morphogenic Properties of the Collagen XVIII Endostatin Domain
Kashi Javaherian, Susan Y. Park, Winfried F. Pickl, Kenneth R. LaMontagne, Robert Tjin Tham Sjin, Stephen Gillies and Kin-Ming Lo

J. Biol. Chem. 2002, 277:45211-45218.
doi: 10.1074/jbc.M206358200 originally published online September 16, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M206358200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 11 of which can be accessed free at http://www.jbc.org/content/277/47/45211.full.html#ref-list-1