ANGPTL3 Stimulates Endothelial Cell Adhesion and Migration via Integrin \(\alpha_v\beta_3\) and Induces Blood Vessel Formation in Vivo*

The angiopoietin family of secreted factors is functionally defined by the C-terminal fibrinogen (FBN)-like domain, which mediates binding to the Tie2 receptor and thereby facilitates a cascade of events ultimately regulating blood vessel formation. By screening expressed sequence tag data bases for homologies to a consensus FBN-like motive, we have identified ANGPTL3, a liver-specific, secreted factor consisting of an N-terminal coiled-coil domain and the C-terminal FBN-like domain. Co-immunoprecipitation experiments, however, failed to detect binding of ANGPTL3 to the Tie2 receptor. A molecular model of the FBN-like domain of ANGPTL3 was generated and predicted potential binding to integrins. This hypothesis was experimentally confirmed by the finding that recombinant ANGPTL3 bound to \(\alpha_v\beta_3\) and induced integrin \(\alpha_v\beta_3\)-dependent haptotactic endothelial cell adhesion and migration and stimulated signal transduction pathways characteristic for integrin activation, including phosphorylation of Akt, mitogen-activated protein kinase, and focal adhesion kinase. When tested in the rat corneal assay, ANGPTL3 strongly induced angiogenesis with comparable magnitude as observed for vascular endothelial growth factor-A. Moreover, the C-terminal FBN-like domain alone was sufficient to induce endothelial cell adhesion and in vivo angiogenesis. Taken together, our data demonstrate that ANGPTL3 is the first member of the angiopoietin-like family of secreted factors binding to integrin \(\alpha_v\beta_3\) and suggest a possible role in the regulation of angiogenesis.

The growth of new blood vessels is a prerequisite during normal physiological processes of embryonic and postnatal development. However, proliferation of new blood vessels from preexisting capillaries, a process termed angiogenesis, also plays a key role in the progression of solid tumor growth, diabetic retinopathies, psoriasis, inflammation, and rheumatoid arthritis (1). Angiogenesis not only is dependent on secreted factors like VEGF\(^2\) or the angiopoietins, which bind to ligate their respective tyrosine kinase receptors expressed on endothelial cells, but is also influenced by cell adhesion molecules binding to their ligands present within the extracellular matrix (2). Inactivation of various genes encoding specific cell adhesion molecules or administration of function blocking antibodies targeting cell adhesion molecules in various animal models resulted in profound inhibitory effects on the angiogenic response of endothelial cells (3).

The integrin family of cell adhesion molecules are two-way signaling receptors responsible for the attachment of cells to the extracellular matrix and for cell-cell interactions that underlie immune responses, tumor metastasis, and progression of atherosclerosis and thrombosis. This family is composed of over 15 \(\alpha\) and eight \(\beta\) subunits expressed in at least 22 different \(\alpha\beta\) heterodimeric combinations. Among these, a combination of six \((\alpha_\beta_2, \alpha_\beta_3, \alpha_\beta_4, \alpha_\beta_5, \alpha_\beta_6, \alpha_\beta_1, \text{ and } \alpha_\beta_7)\) has been implicated in angiogenesis (4, 5). Integrins facilitate cellular adhesion to and migration on extracellular matrix proteins located within the intercellular spaces and basement membranes. Some integrins were shown to bind to FBN-like domains encoded by various ligands found within the extracellular matrix (6, 7). Integrin \(\alpha_\beta_3\) binds to a wide variety of extracellular matrix proteins including vitronectin, fibronectin, fibrinogen, laminin, collagen, von Willebrand factor, osteopontin, and a fragment of MMP2 (PEX) among others (for a review, see Ref. 8). Despite its promiscuous ligand binding behavior, \(\alpha_\beta_3\) is not widely expressed in adult tissues and was found on some vascular, intestinal, and uterine smooth muscle cells (9). This receptor was also found on activated leukocytes, on macrophages and osteoclasts, where it regulates bone resorption (10). Most prominently, \(\alpha_\beta_3\) becomes up-regulated on endothelial cells exposed to hypoxia and cytokines such as VEGF-A (11, 12) and was found to be overexpressed on tumor vasculature or in atherosclerotic arteries (13).

ANGPTL3 was previously found to be expressed in a liver-specific manner during development and in adults (14), and more recently, it was found to be involved in the regulation of serum lipid levels in mice (15). We independently cloned ANGPTL3 based on sequence homologies with the FBN-like domains located within the carboxyl terminus of the angiopoietins (16). Since ANGPTL3 did not bind to the angiopoietin receptor Tie2, we tried to identify potential candidate receptors.

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1 The abbreviations used are: VEGF, vascular endothelial growth factor; BSA, bovine serum albumin; PMA, phorbol 12-myristate 13-acetate; FAK, focal adhesion kinase; HUVEC, human umbilical venous endothelial cell; HMVEC, human microvascular vein endothelial cell; CS-C, Cell System complete; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; FBN, fibrinogen; ARP1, angiopoietin-related protein 1; VN, vitronectin; Ang1 and -2, angiopoietin 1 and 2, respectively; MAPK, mitogen-activated protein kinase; hANGPTL3, human ANGPTL3; mANGPTL3, murine ANGPTL3.

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Fig. 1. ANGPTL3 and Ang2 but not ARP1 bind to HMVECs. A, domain structure comparison between angiopoietins 1 and 2, full-length ANGPTL3, and FBN-ANGPTL3. B, FACS chromatograms of HMVECs incubated with conditioned medium from transiently transfected CHO cells containing a gD epitope-tagged version of human angiopoietin 2, ARP1, or ANGPTL3 or control medium. For relative ligand expression levels, see Fig. 2.

by taking advantage of the structural information available for other, related FBN-like domains. The three-dimensional structure of the C terminus of the γ-chain of human fibrinogen has been solved by x-ray crystallography (17), and we have used this structure information as a template to build a three-dimensional model of the FBN-like domain of ANGPTL3. This model strongly suggested members of the integrin family of cell adhesion molecules as potential candidates for binding. Cell-based assays with recombinant proteins and direct protein-binding experiments revealed that ANGPTL3 was binding to αvβ3 and induced endothelial cell migration and adhesion, which was potently abolished by the presence of function blocking antibody-targeting integrin αvβ3. The robust induction of blood vessel growth by ANGPTL3 in the rat corneal angiogenesis assay revealed that this liver-specific, secreted protein is a novel angiogenic factor.

EXPERIMENTAL PROCEDURES

Reagents and Cell Culture—BSA, FMA and poly-l-lysine were purchased from Sigma and Genetin; GRGDP and GRGESP peptides were from Invitrogen. Purified monoclonal anti-integrin antibodies JBS5 (anti-αvβ3), LM609 (anti-αvβ3), and P1F6 (anti-αvβ3) were from Chemicon. Anti-phospho-FAK (pY397) was obtained from BIOSOURCE International, and anti-FAK was from BD Transduction Laboratories. Anti-phospho-p42/44 MAPK (pY202/204), anti-p42/44 MAPK, anti-phospho-Akt (pS473), anti-Akt antibodies, and horseradish peroxidase-conjugated secondary antibodies were purchased from New England Biolabs. ECL Plus reagent and Hyperfilm were obtained from Amersham Biosciences. Human umbilical venous endothelial cells (HUVECs) and human microvascular vein endothelial cells (HMVECs) and Cell System complete (CS-C) medium were purchased from Cell System (Kirkland, WA). Cells were maintained in CS-C complete medium containing 10% fetal bovine serum and mitogens, according to the recommendations of the supplier. HUVECs and HMVECs were used at passage 6 or below and collected from confluent culture dishes. The αvβ3-overexpressing 293 cell line was generated as reported previously (18). Other integrin-overexpressing lines were generated using similar methods by transfecting the appropriate cDNAs together with a G418 resistance gene into 293 cells. Multiple rounds of FACS, using subunit-specific antibodies, were carried out to select clones expressing >106 receptors/cell. All 293 lines were grown in Dulbecco's modified Eagle's medium (high glucose; Invitrogen) supplemented with 10% fetal bovine serum and Genetin (400 μg/ml). All cell lines were cultivated in a humidified atmosphere containing 5% CO2 at 37°C.

Integrin αvβ3 Binding Enzyme-linked Immunosorbent Assay—The ability of ANGPTL3 to bind to αvβ3 was evaluated in 96-well plates (Maxisorp; Nunc). Plates were coated overnight at 4°C with the indicated concentrations of ANGPTL3, fibronectin (Calbiochem), or BSA in PBS. The plates were blocked for 1 h at room temperature with 0.5% BSA in PBS. After washing the plates six times with wash buffer (0.05% Tween 20 in PBS), the indicated concentrations of αvβ3 in assay buffer (50 mM Tris, pH 7.4, 0.5% BSA, 0.05% Tween 20, 1 mM MnCl2, 50 μM CaCl2, 50 μM MgCl2, 100 mM NaCl) were added. The plates were allowed to incubate for 2 h; subsequently, they were washed six times with wash buffer. The bound αvβ3 was detected with mouse monoclonal anti-β3 (clone 4B12; Genentech) labeled with horseradish peroxidase. The plates were incubated for 2 h and then washed six times with wash buffer. The bound peroxidase activity was assessed with tetramethylbenzidine as substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The results are expressed as A450.

Cloning, Expression, and Purification of Recombinant Proteins—Human ANGPTL3 was cloned into the eukaryotic expression vector pRK5tkNEO and the baculovirus vector pHIF, a derivative of pVL1393 purchased from PharMingen. The gD epitope-tagged murine ortholog as well as a fusion protein consisting of the fibrinogen domain of ANGPTL3 (amino acids 241–454) fused to a FLAG epitope-tagged leucine zipper domain were generated as described previously (20, 21). In all constructs encoding a gD epitope, amino acids 1–17 of the native
protein were replaced with the gD epitope encoding a signaling sequence as described previously (20). Proteins were expressed in Chinese hamster ovary (CHO) cells (see Fig. 4A) and purified by using anti-gD or anti-FLAG affinity columns, respectively. Briefly, plasmid DNA was cotransfected with BaculoGold DNA (PharMingen) into Sf9 cells using Lipofectin (Invitrogen). After 4 days, the cells were harvested, 500 μl of the supernatant was used to infect 2 × 10⁶ Sf9 cells, and baculovirus was amplified. After 72 h of amplification, the cells were harvested, and 10 ml of the supernatant was used to infect 7.5 × 10⁶ H5 cells/ml for 40 h. After harvesting and filtration through a 0.45-μm cellulose ace-

![Homology modeling of the fibrinogen domain of ANGPTL3.](image)

**Fig. 3.** Homology modeling of the fibrinogen domain of ANGPTL3. A, Superimposition of the x-ray structure of the C terminus of the γ-chain of human fibrinogen (3FIB) in white and the modeled structure of the FBN-like domain of ANGPTL3 in green. α-Helices are shown as cylinders, and β strands are shown as arrows. Regions that differ in both structures are labeled. Regions shown to be involved in binding to integrins in FBN and structurally conserved in FBN-ANGPTL3 are highlighted in yellow. B, sequence alignment of the C terminus of the γ-chain of human fibrinogen (3FIB) and the FBN-like domain of ANGPTL3 and human angiopoietins 1, 2, and 4. Hydrophilic and charged residues are displayed in blue, and aromatic/hydrophobic residues are shown in orange. The consensus is shown below the alignment, with conserved hydrophilic/charged and aromatic/hydrophobic mutations marked as blue and orange squares, respectively. Numbering corresponds to the 3FIB x-ray structure. C, sequence similarity between the C-terminal γ-chain of human fibrinogen (3fib) and the FBN-like domain of human ANGPTL3, CDT6 (cornea-derived transcript 6), and angiopoietins 1, 2, and 4. Percentage identities are shown in gray, and percentage similarities are shown in yellow.

**Fig. 4.** ANGPTL3 is a secreted glycoprotein. A, Coomassie-stained SDS-polyacrylamide gel of immunoaffinity-purified human gD-hANGPTL3 (60.1 kDa) purified from baculovirus extracts. B, silver-stained SDS-polyacrylamide gel of immunoaffinity-purified murine gD-mANGPTL3 (57.9 kDa) purified from transiently transfected CHO cells. C, comparison of molecular weights between recombinant gD-hANGPTL3 protein with (+) or without (−) peptide-N-glycanase F (PNGase-F) treatment. Western blots were incubated with a mouse monoclonal anti-gD antibody.
tate filter, the supernatant was purified. Mouse ANGPTL3 and human FBN-ANGPTL3 were overexpressed in CHO cells in large scale transient transfection process. Cells were grown in fully automated bioreactors using F-12/Dulbecco's modified Eagle's medium-based media supplemented with Ultra-Low IgG serum (Invitrogen) and Primatone HS (Sigma). The culture was maintained for 7–12 days until harvest. Human ANGPTL3 was purified from the supernatants of baculovirus-infected insect cells grown in suspension utilizing immunoaffinity chromatography. The column was generated by coupling anti-gD Fab to glycophase-CPG (controlled pore glass). The clarified (1000 × g for 5 min and then 0.2-μm filtered) medium was loaded overnight at 4 °C. The column was washed with PBS until the absorbance at 280 nm of the effluent returned to baseline and eluted with 50 mM sodium citrate at pH 3.0. The eluted protein was dialyzed (Spectra-pore; molecular weight cut-off, 10,000) against 1 mM HCl and frozen at −70 °C. Transiently expressed CHO cultures were clarified and concentrated using a 10,000 molecular weight cut-off membrane (Amicon). This volume was passed over an anti-gD Fab coupled to glycophase-CPG column as

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**FIG. 5.** Haptotactic adhesion of 293 cells overexpressing αβ₃-integrin to ANGPTL3 and direct binding of recombinant hANGPTL3 to αβ₃. A. adhesion of 293 cells overexpressing either integrin α₄β₁, α₃β₁, α₃β₃, or α₃β₅ was tested in microtiter plates coated with 20 μg/ml hANGPTL3 or BSA. Cells were allowed to adhere at 37 °C and quantified after 4 h. B, comparison of 293 cells expressing αβ₃ adhering to hANGPTL3, fibronectin (FN), and VN coated on plates at the concentrations indicated. C, recombinant hANGPTL3, hAng1*, and fibronectin were coated at the indicated concentrations. A₄₅₀ was determined in an enzyme-linked immunosorbent assay after incubation of the plates with 100 μg/ml recombinant αβ₃, followed by secondary antibodies binding to αβ₃. Data shown represent means ± S.D. of one representative experiment run in duplicates from three independent experiments.
previously described for human ANGPTL3. The eluted pool was diluted with 10 mM sodium acetate (pH 5.0) to a conductivity of 10 mS and loaded onto S Sepharose Fast Flow (Amersham Biosciences). The column was washed with 10 mM sodium acetate, pH 5.0, until the absorbance of the effluent at 280 nm returned to baseline and eluted with a 20-column volume gradient 0–0.5 M NaCl in 10 mM sodium acetate, pH 5.0. The fractions that eluted at 0.45–0.5 M NaCl, containing mouse ANGPTL3, were further purified utilizing reverse phase C-4 chromatography (Vydac). The fractions were acidified with 0.1% trifluoroacetic acid and loaded on the C-4 column and then eluted with a 0–100% acetonitrile, 0.1% trifluoroacetic acid gradient. The mouse mANGPTL3 eluted at 67% acetonitrile, was lyophilized, and was stored at -70°C.

The identities of the purified proteins were verified by N-terminal sequence analysis. The lipopolysaccharide concentration was verified using commercial kits and determined to be 5 EU/mg for all human or murine ANGPTL3 preparations.

Molecular Modeling of the FBN-like Domain of ANGPTL3—To build the FBN-ANGPTL3 model, a sequence-structure alignment between our sequence and several FBN domain structures was performed by using ClustalW (22) and threading (ProCeryon Biosciences Inc.). From this alignment, the γ-fibrinogen x-ray structure at 2.1-Å resolution (3FIB Protein Data Bank entry code) was chosen as a template structure for model construction. The program PROCHECK (23) was used to assess the geometric quality of the model, which was of above average stereochemical quality when compared with the reference data base of structures deposited in the Protein Data Bank. The final FBN-ANGPTL3 model had a root mean square deviation of 1.95 Å for all Cα atoms when compared with the template. The ANGPTL3 FBN-like domain was modeled by using Insight II (version 98.0; MSI, San Diego, California).

Peptide:N-Glycanase F, Coimmunoprecipitation, Western Blot, and FACS—The glycosylation status of the recombinant ANGPTL3 was determined with peptide:N-glycanase F treatment according to the manufacturer’s protocol (New England Biolabs). Purified protein (50 ng) was electrophoresed through SDS-polyacrylamide gel (10% Tris/ glycine; Invitrogen) and electrotransferred to nitrocellulose membranes (Invitrogen, CA) using standard procedures. The membrane was blocked by incubation in 5% (w/v) instant nonfat milk powder in PBS and incubated overnight at 4°C with 1 μg/ml monoclonal anti-gD (clone 5B6.K6) antibody in blocking buffer. The membranes were washed with PBS plus 0.05% Tween 20 and subsequently incubated with horseradish peroxidase-coupled donkey anti-mouse antibodies (Jackson Immuno...
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RESULTS

ANGPTL3 Binds to Endothelial Cells via Receptors Distinct from Tie1 or Tie2—The sequences for both human and mouse ANGPTL3 have been published previously (14). In analogy to the angiopoietins, ANGPTL3 is a secreted factor consisting of an N-terminal signal peptide, followed by a coiled-coil domain and a C-terminal FBN-like domain (Fig. 1A). First, we tested whether ANGPTL3 binds to primary endothelial cells known to express the Tie2 receptor by exposing HMVECs to conditioned media derived from transiently transfected 293 cells. The expression vectors encoded G D epitope-tagged versions of ANGPTL3 and Ang2, which served as a positive control for Tie2 binding. As negative control, we included conditioned media containing ARPI (28), an angiopoietin-like ligand encoding a signal peptide, an N-terminal coiled-coil, and a C-terminal FBN-like domain. As shown in Fig. 1B, Ang2 and ANGPTL3 bound to HMVECs under conditions in which ARPI binding was not detectable. These findings demonstrated that binding of ANGPTL3 to endothelial cells was specific and suggested the presence of receptors on endothelial cells binding to ANGPTL3.

To test whether Tie2 or Tie1, an orphan receptor with high sequence homology to Tie2, were interacting with ANGPTL3, we conducted co-immunoprecipitation experiments with transiently transfected 293 cells expressing Ang1 or -2, ANGPTL3, or ARP1 with full-length receptor constructs for either receptor. Neither Tie1 nor Tie2 bound to ANGPTL3 under experimental conditions that allowed Ang1 or -2 binding to Tie2 (Fig. 2). Similar results were obtained when co-transfection experiments were conducted with endothelial cells (data not shown).

Molecular Modeling of FBN-ANGPTL3 and Prediction of Integrin Binding Domains—As shown in Fig. 3, B and C, sequence comparison revealed a 55–65% sequence identity between the FBN-like domains from Ang1 and Ang2 but only 37–39% homology in the cross-comparison with ANGPTL3 or CD147, respectively. The latter is a recently identified member of the angiopoietin-like family of secreted factors consisting of a coiled-coil and an FBN-like domain (29). Such high levels of sequence homology between the FBN-like domains of ANGPTL3 and the angiopoietins suggested that other receptor systems than Tie2 might be involved in ANGPTL3 binding to endothelial cells.

To further investigate the molecular mechanisms, by which ANGPTL3 binds to endothelial cells, we built a model of the
FBN-like domain present within ANGPTL3 by using structural information provided by x-ray crystallographic studies on related FBN domains and by homology modeling techniques. The FBN-like domain of ANGPTL3 shares 39.4% sequence identity with the C terminus of the γ-chain of human fibrinogen (Fig. 3, B and C). The FBN domain has a unique fold consisting of three well defined domains: an N terminus domain formed by a two-stranded antiparallel β-sheet flanked by a short helix; a central domain formed by a five-stranded antiparallel β-sheet with two short helices and a hairpin loop aligned against one of its faces; and a third domain that is composed predominantly of loops (Fig. 3A). The overall fold of the FBN domain is conserved in FBN-ANGPTL3, with some differences in the loop regions 220–224, 289–306, and 357–363 (Fig. 3A).

Studies on the human fibrinogen γ chain led to the identification of two regions involved in binding to the integrin α₄β₂ (Mac-1, CD11b), an integrin predominantly expressed on leukocytes (6). Both regions, separated in terms of linear amino acid sequence, form two adjacent antiparallel β-strands in the three-dimensional structure of the FBN domain. A different region within the fibrinogen γ-chain and tenascin-C was found to be involved in binding to integrin α₄β₂ (7). The FBN-like domain of ANGPTL3 and the fibrinogen γ-chain share a high degree of structural similarity in those regions (indicated in yellow in Fig. 3A), suggesting integrins as potential candidate receptors.

To test this model, we generated recombinant human and murine ANGPTL3 protein preparations encoding an amino-terminal gD epitope (20). Both full-length recombinant proteins migrated as single bands at the expected molecular size of 60.1 kDa (human) and 57.9 kDa (mouse). In the murine preparations, we occasionally observed a smaller band migrating at a molecular size of about 30 kDa. Microsequencing revealed this band to be an N-terminal cleavage product cleaved at leucine 294. Whether this N-terminal fragment reflects an in vitro artifact caused by the transient CHO cell expression procedure or whether it represents a regulatory mechanism controlling ligand activity remains to be determined. However, we have not observed any correlation between the in vitro activity and the relative amounts of the cleavage product present in the various preparations tested (data not shown).

In contrast to Ang1 and -2, which formed disulfide-linked oligomeric complexes under nonreducing conditions (16), only marginal amounts of ANGPTL3 oligomerization product were found when protein preparations were analyzed by gel electrophoresis in the absence of reducing agents (data not shown). The glycosylation status of the recombinant hANGPTL3 was determined with peptide:N-glycanase F digestion. The increase in mobility of the hANGPTL3 band upon incubation with peptide:N-glycanase F indicated that the recombinant protein was glycosylated (Fig. 4C), similar to previous findings for the angiopoietins (29, 30).

Cells Expressing α₄β₂ Adhered to ANGPTL3-coated Dishes, and a Neutralizing Antibody to α₄β₂ Completely Inhibits Endothelial Cell Adhesion to ANGPTL3—We tested a series...
of 293 cell lines stably expressing different integrin heterodimers including IIbIIIa (αIIbβ3), αvβ3, αvβ5, and αvβ6, for their adherence to culture dishes coated with recombinant ANGPTL3, BSA, or prototypic integrin ligands such as fibronectin and vitronectin (VN). Among the cell lines tested, cells expressing αvβ3 displayed a marked increase in adherence to ANGPTL3 (Fig. 5A) 4 h after cell plating. Cell adhesion correlated with coating concentrations, and at 20 μg/ml cell adhesion to ANGPTL3 was comparable with the levels obtained for fibronectin and vitronectin, two prototypic integrin ligands (Fig. 5B). A similar, coating concentration-dependent increase in binding was observed when purified αvβ3 was incubated with surface-coated, recombinant hANGPTL3, and binding was assessed by enzyme-linked immunosorbent assay (Fig. 5C). In agreement with previous reports (31), the structurally related hAng1* failed to bind to recombinant αvβ3, suggesting selective integrin binding specificity by proteins containing various FBN-like domains. Primary HMVECs displayed a concentration-dependent increase in cell adhesion assay when plated on hANGPTL3-coated plates 4 h after cell plating (Fig. 6A). In general, HMVEC adhesion to ANGPTL3-coated dishes was between 20 and 50% relative to the levels observed for fibronectin-coated plates. These lower levels when compared with data derived from 293 cells may reflect the presence of additional integrins on endothelial cells binding to fibronectin.

To test whether the fibrinogen-like domain alone was sufficient to induce cell adhesion, we tested recombinant ANGPTL3-FBN-like protein in the endothelial cell adhesion assay. To allow for protein oligomerization, we replaced the coiled-coil domain with a leucine zipper domain known to induce protein oligomerization. In addition, the construct encoded a C-terminal FLAG-epitope for protein purification purposes (Fig. 1A). As demonstrated in Fig. 6B, FBN-hANPTL3 alone was sufficient to induce HMVEC adhesion. However, in order to obtain similar levels of cell binding, culture dishes needed to be coated with about 4-fold higher concentrations compared with the native, full-length protein. The reason for the lower cell adhesion activity displayed by the fibrinogen-only construct remains unclear. It may reflect conformational changes or additional interactions between the cell surface and the coiled-coil domain not present in the FBN-hANPTL3 construct.

To test to what extent such haptotactic response of endothelial cells to ANGPTL3 is mediated by αvβ3, we added a series of function-blocking antibodies for various integrins to the adhesion assay. As shown in Fig. 6C, αvβ3-specific antibody completely blocked HMVEC adhesion. In contrast, antagonistic antibodies for αvβ3 and αvβ5 of the same isotype did not impair adhesion to ANGPTL3 (20 μg/ml)-coated plates (Fig. 6C).

Integrin αvβ3 was found to recognize some of its ligands in the context of the RGD adhesive sequence (3). Consistent with the notion that the FBN-like domain of ANGPTL3 does not encode such RGD sequence, the addition of RGD peptides only partially abolished HMVEC adhesion under conditions where control RGE peptides displayed no effect. As a general control abrogating integrin-ligand binding, EDTA (10 μM) was added to the cell binding conditions. Such interference with the integrin dependence on divalent cations completely abolished endothelial cell adhesion to ANGPTL3. However, while these experiments point out αvβ3 as a candidate receptor, they do not rule out the possibility that other, yet untested integrins may contribute to ligand binding in other experimental or cellular contexts.

Endothelial Cells Display Haptotactic Migratory Responses to ANGPTL3—Another hallmark of integrin-mediated cellular effects is the migratory response of cells in response to ligand stimulation. To assess whether ANGPTL3 induces migration of HMVECs, we applied the migration assay using transwell chambers. 16–20 h after stimulation with ANGPTL3, we observed a >2.5-fold increase in cell migration when compared with BSA control (Fig. 6D). Such induction of endothelial cell migration was most prominently detected when recombinant protein was coated to the membrane surface and to a lesser extent when the protein was added to the medium (data not shown). This is a characteristic feature of proteins exerting their effect in a haptotactic manner (33). The migratory response of HMVECs was abolished by the presence of an antagonistic antibody to αvβ3 but not by the control function-blocking antibody targeting αvβ5 of the same isotype (Fig. 6D).

In summary, ANGPTL3 potently induced haptotactic adhesion and migration of primary human endothelial cells; both responses were significantly inhibited by the presence of antagonistic antibodies to αvβ3. Similar to the findings for Ang1 and -2 (34), ANGPTL3 failed to induce proliferation of primary human endothelial cells including HUVECs and HMVECs grown in 1% FCS-containing medium (data not shown).

Analysis of Signal Transduction Pathways Engaged by ANGPTL3 in Endothelial Cells—Activation of integrins leads to stimulation of numerous intracellular signal transduction pathways including FAK, mitogen-activated protein kinase (MAPK), and Akt/protein kinase B (35), resulting in characteristic cytoskeletal rearrangements. In order to study such events in HMVEC, we analyzed the phosphorylation status of candidate signaling molecules at various time points after exposure to ANGPTL3 (Fig. 7). FAK phosphorylation was most pronounced when HMVECs were stimulated with VN, the prototypic ligand for αvβ3. A significant increase in phosphorylation was observed as early as 60 min after stimulation by ANGPTL3, and the levels obtained after 4 h were comparable with VN-stimulated cells. As negative control, cells were seeded on poly-l-lysine-coated dishes, conditions that reportedly do not induce MAPK and FAK phosphorylation (32). We found a strong -fold induction of MAPK phosphorylation in cells exposed to ANGPTL3, which exceeded the levels obtained for VN. Furthermore, a strong increase in the phosphorylation status of Akt/protein kinase B at Ser473 was observed as early as 60 min after seeding endothelial cells on ANGPTL3-coated plates, and this response was comparable with VN stimulation. In contrast, only weak levels were detected in cells grown on poly-l-lysine-coated plates. Thus, endothelial cell adhesion induced by ANGPTL3 stimulates FAK, MAPK, and Akt/protein kinase B phosphorylation with similar magnitude as observed for vitronectin; however, there were differences with regard to the kinetics of these responses.

ANGPTL3 Induces Angiogenesis in the Rat Cornea—To test whether ANGPTL3 was capable of inducing neoangiogenesis in vivo, we implanted hydron pellets containing murine and human ANGPTL3 (500 ng), human VEGF (100 ng), and control compounds separately or in combination into rat corneas. Both murine and human ANGPTL3 (500 ng) potently induced angiogenesis to comparable levels as obtained for VEGF (50 ng) 6 days after pellet implantation (Fig. 8, A–C). In the combination treatment with VEGF, we observed additive but not synergistic effects (Fig. 8D), probably reflecting interdependent signal transduction pathways engaged by both ligands (2). In agreement with previous reports (36), recombinant murine Ang2 failed to induce an angiogenic response in this assay (Fig. 7F). Finally, the C-terminal fibrinogen-like domain, fused to an N-terminal leucine zipper motif, potently induced neoangiogenesis in this model. Histological analysis of corneal sections did not reveal any significant inflammatory events in all groups tested (data not shown). These findings are consistent with our
observation that recombinant ANGPTL3 failed to bind to the integrins LFA-1 (CD11a) or Mac-1 (CD11b, data not shown), which are strongly expressed on activated leukocytes. Thus, ANGPTL3 is to our knowledge the first member within the angiopoietin-like family of secreted factors capable of inducing a robust angiogenic response in vivo independently from other angiogenic factors like VEGF.

**DISCUSSION**

The Fibrinogen-like Domain of ANGPTL3 Is Sufficient for Integrin Binding and Induction of Angiogenesis—The angiopoietins, along with their respective cell surface receptor tyrosine kinase Tie2, comprise a well-characterized family of angiogenic factors (for a review, see Ref. 37). Unlike other angiogenic cytokines, which induce receptor tyrosine phosphorylation after receptor binding and ligation, angiopoietins are not directly mitogenic for endothelial cells. Recently, Carlson et al. (32) demonstrated that angiopoietin 1 and 2 can serve as substrate for cell adhesion by binding to the integrins β1 and αβ3 (32), and similar findings were reported for the latent form of tumor growth factor-β (38). These studies were conducted using full-length protein preparations and did not identify the structural domains involved in integrin binding and activation. The morphological changes of endothelial cells exposed to Ang1 and Ang2, as well as the signal transduction pathways engaged by both ligands, varied significantly. Whereas either ligand stimulated HUVEC adhesion and MAPK activation, only Ang1 induced marked cell spreading, cell migration, FAK activation, and formation of actin stress fibers. Due to the fact that HUVEC cells express both receptor systems for angiopoietins, integrins and Tie2, it is unclear to what extent these signaling pathways are regulated by each receptor system separately. Since ANGPTL3 does not bind to Tie2, we were able to specifically analyze a Tie2-independent signal transduction pathway in endothelial cells. However, due to the strong interference of the antagonistic antibody for αβ3 with endothelial cell adhesion (Fig. 5), we were unable to assess whether the haptotactic signaling events in endothelial cells were solely mediated by αβ3. In conclusion, the FBN-like domain of ANGPTL3, when fused to a leucine zipper motif, was sufficient to induce neangiogenesis, suggesting the FBN-like domain to be sufficient to induce angiogenesis.

**Potential Vascular Functions of ANGPTL3 Binding to Integrin αβ3**—ANGPTL3 expression in adults was found to be restricted to the liver, and thus it may represent a liver-specific angiogenic factor regulating vascular responses by binding to αβ3 expressed on the liver endothelium. However, our findings that recombinant ANGPTL3 induced a strong angiogenic response in the rat cornea open the possibility that regulation of angiogenesis at distant sites of its production is part of the biological function. In either scenario, ligation of αβ3 on endothelial cells might subsequently regulate vascular functions associated with αβ3 such as angiogenesis, leukocyte adhesion, transmigration, or metabolite transport through the endothelium. In order to distinguish between systemic and tissue-specific mechanisms, the generation of antibodies and the development of transgenic mouse models may be instrumental.

Angptl3 was recently identified in a positional cloning approach aimed at the identification of an autosomal recessive mutation responsible for low plasma lipid levels in KK/San mice (15). These hypolipidemic mice are derived from KK obese mice, which display a multigenic syndrome of moderate obesity and a diabetic phenotype including hyperinsulinemia, hyperglycemia, and hyperlipidemia. Administration of ANGPTL3 to KK/San mice elicited a rapid increase in circulating plasma lipid levels, suggesting that the suppression of ANGPTL3 function in KK/San mice caused the decrease in plasma triglyceride levels in these mice. While these studies revealed a potential role for ANGPTL3 in lipid metabolism, they did not address the cellular and molecular mechanism by which hyperlipidemia in response to ANGPTL3 was mediated. The short and transient response of mice treated with ANGPTL3 (1 h post-treatment) and the equal expression levels in ApoB mRNA in both mouse strains suggested that ANGPTL3 is not involved in the regulation of high density lipoprotein cholesterol synthesis or catabolism. Moreover, previous observations revealed differences in the secretion rates of triglycerides between the mutant strains. Thus, one of the potential vascular functions of ANGPTL3 may include the regulation of lipid secretion into the circulation. It remains to be tested whether the increase in lipid release or accelerated lipid transport by ANGPTL3 is an αβ3-dependent event.

Circumstantial evidence for a potential role of the vasculature in mediating some aspects of ANGPTL3 biological functions was provided by the observation that KK/San mice displayed a decrease in artherosclerosis. Thus, the identification of αβ3 as a receptor for ANGPTL3 on the vasculature may help in dissecting its role in lipid metabolism and inflammatory diseases.

Our observation that ANGPTL3 potently induced angiogenesis in the rat cornea does not exclude a role in lipid metabolism. As previously shown for leptin, a hormone involved in the regulation of food uptake and lipid metabolism, one factor may exert multiple biologic functions. Similar to ANGPTL3, leptin potently induced neovascularization when tested in the corneal angiogenesis assay, presumably via activation of OB-Rb receptors in the vasculature (39). Thus, ANGPTL3 and leptin might be part of a family of angiogenic molecules that are involved in the regulation of lipid metabolism.

Most of the integrin ligands identified so far are expressed ubiquitously throughout most tissues during development and in adult stages but are subject to activation in a more tissue-restricted manner during many pathological conditions such as tumor growth and/or inflammatory responses (3). It remains to be seen whether ANGPTL3 plays a role during pathologic angiogenesis in the liver or other tissues and whether posttranslational modifications may regulate its activity.

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