Expression of Antisense to Integrin Subunit \( \beta_3 \) Inhibits Microvascular Endothelial Cell Capillary Tube Formation in Fibrin

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\( \alpha_\beta_3 \) antagonists are potent angiogenesis inhibitors, and several different classes of inhibitors have been developed, including monoclonal antibodies, synthetic peptides, and small organic molecules. However, each class of inhibitor works by the same principle, by blocking the binding of ligands to \( \alpha_\beta_3 \). In an effort to develop an \( \alpha_\beta_3 \) inhibitor that down-regulates the actual level of \( \alpha_\beta_3 \), we developed an antisense strategy to inhibit \( \alpha_\beta_3 \) expression \textit{in vitro}. \( \beta_3 \) antisense expressed in endothelial cells specifically down-regulated \( \alpha_\beta_3 \) and inhibited capillary tube formation, with the extent of down-regulation correlating with the extent of tube formation inhibition. This inhibition was matrix-specific, since tube formation was not inhibited in Matrigel. These findings support the notion that \( \alpha_\beta_3 \) is required for an essential step of angiogenesis in fibrin, namely capillary tube formation. These results suggest that pseudogenetic inhibition of \( \beta_3 \) integrins using antisense techniques may ultimately provide a therapeutic means to inhibit angiogenesis \textit{in vivo}.

Angiogenesis is the process of blood vessel development from preexisting vessels. In the normal adult, little angiogenesis occurs except as part of wound healing and during certain events in reproduction, including the menstrual cycle and embryonic implantation (1, 2). Although aberrant angiogenesis is a hallmark of disorders such as solid tumor growth and metastasis (1, 2), diabetic retinopathy (3, 4), rheumatoid arthritis (5), atherosclerosis (6), and restenosis following angioplasty (7), these processes have clinical implications (1, 2). Although aberrant angiogenesis is a hallmark of disorders such as solid tumor growth and metastasis (1, 2), diabetic retinopathy (3, 4), rheumatoid arthritis (5), atherosclerosis (6), and restenosis following angioplasty (7), pharmacologic stimulation of angiogenesis can promote development of a beneficial collateral circulation around occluded blood vessels, including coronary arteries (8). Therefore, much research is currently under way to identify methodologies to modulate angiogenesis.

Endothelial cell adhesion molecules are attractive targets to inhibit angiogenesis. \( \beta_3 \) integrins (9–11), integrin \( \alpha_\beta_3 \), and integrin \( \alpha_\beta_3 \) (12) have all been implicated in angiogenesis, although the role of integrin \( \alpha_\beta_3 \) in angiogenesis has been a subject of controversy. Angiogenic blood vessels in humans and other species express \( \alpha_\beta_3 \), but normal quiescent vasculature expresses little to no \( \alpha_\beta_3 \) (13). Mouse knock-out studies, however, have shown convincingly that \( \alpha_\beta_3 \) is not required for angiogenesis during embryonic development (14, 15), and mutations in the \( \beta_3 \) subunit of individuals with Glanzmann’s thrombasthenia cause no apparent problems with angiogenesis (16), although these genetic findings do not rule out the possibility of compensation during development by functionally redundant adhesion molecules. In contrast to these genetic data, compelling pharmacologic data shows that monoclonal antibody and peptide antagonists of \( \alpha_\beta_3 \) are potent inhibitors of angiogenesis in animals and humans (13, 17–21). Recent findings suggest that some of the actions of \( \alpha_\beta_3 \) may be mediated by its coupling to receptors for growth factors, including platelet-derived growth factor (22) and vascular endothelial growth factor (23). A chimeric derivative (24) of a monoclonal antibody directed against \( \alpha_\beta_3 \), LM609 (25), has been used in clinical trials as an angiogenesis inhibitor. Synthetic peptide inhibitors of \( \alpha_\beta_3 \) have been tested as angiogenesis inhibitors to prevent retinopathy (3, 4).

To date, \( \alpha_\beta_3 \) antagonists have been primarily antibodies (24), peptides (3, 4), and small organic molecules (26, 27). However, each class of antagonist binds directly to \( \alpha_\beta_3 \), which has the potential to cause unintended cell signaling (28). The use of RNA antisense as an antiangiogenic strategy has not yet been fully explored. While no drug is entirely selective, antisense has a theoretical advantage over antibodies, inhibitory peptides, or organic molecules in that the drug does not bind directly to the receptor. Furthermore, \( \alpha_\beta_3 \) expression can theoretically be targeted specifically, unlike the pleiotypic effects seen with certain cytokines such as transforming growth factor \( \beta \) or interferon \( \gamma \) (29). Integron subunits such as \( \alpha_\delta \) and \( \beta_3 \) have been successfully targeted \textit{in vitro} with RNA antisense directed against different regions of the mRNA (30, 31). RNA antisense against other target molecules has been used in clinical trials to treat such diverse conditions as ovarian cancer, Crohn’s disease, and retinal damage caused by cytomegalovirus (32). In this paper, we present evidence that the expression of endogenous antisense RNA directed against the integrin \( \beta_3 \) subunit inhibits endothelial cell capillary tube formation \textit{in vitro}.

**EXPERIMENTAL PROCEDURES**

Construction of \( \beta_3 \) Sense and Antisense Expression Vectors—Full-length human \( \beta_3 \) cDNA was used to generate four different \( \beta_3 \) antisense and two sense constructs by PCR. A 130-bp antisense fragment of \( \beta_3 \) cDNA was used to generate four different \( \beta_3 \) antisense and two sense constructs by PCR. 1 A 130-bp antisense fragment of \( \beta_3 \) cDNA was used to generate four different \( \beta_3 \) antisense and two sense constructs by PCR. The abbreviations used are: PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; bFGF, basic fibroblast growth factor; bp, base pair(s); PBS, phosphate-buffered saline.

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erated using forward primer 5'-CGC GGA TCC GAT GGC CCT GCG GTC ACG-3' and reverse primer 5'-CGG GAT CCC GAT CCT CCG CAG TTC GAT GGA GAA-3'. A 240 bp sense fragment of the 5' from nucleotides 180–419 was generated using forward primer 5'-CGG GAT CCC GAT CCT CCG CAG TTC GAT GGA GAA-3' and reverse primer 5'-CGC GGA TCC GAT GGC CCT GCG GTC ACG-3'.

The human dermal microvascular endothelial cell line (HMEC-1) was obtained from Dr. Edwin W. Ades (Centers for Disease Control and Prevention, Atlanta, GA). HMEC-1 cells were grown in MCDB-131, 10% fetal bovine serum, 2 mM L-glutamine, 200 kallikrein-inactivating units/ml aprotinin and 30 ng/ml bFGF (R & D Systems). The control for loading and transfer, parallel blots were stained for total protein with 0.1% Ponceau S, 1% acetic acid and scanned using an Epson Perfection 636 scanner.

**Western Blot Analysis**—Cell extracts were prepared from HMEC-1 cells by solubilizing 10-cm plates of cells at 4 °C with 1 ml of 150 mM NaCl, 10 mM 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate, 10 mM Hepes, pH 7.4, containing 1 mM leupeptin, 0.1 mM n-ethylmaleimide, 1 μM pepstatin, 0.1 mM phenylmethylsulfonyl fluoride. Protein concentration was determined using a bicinchoninic acid assay (Pierce) and equal amounts of extracted cell proteins were loaded on 7.5% polyacrylamide gels (39). Gels were transferred to nitrocellulose (40) at 0.65 V/cm for 4 h at 4 °C. The nitrocellulose blots were probed with either anti-αv, rabbit polyclonal antiserum (AB 1950; Chemicon) or anti-β3 rabbit polyclonal antiserum (AB 1952; Chemicon), using a goat anti-rabbit Ig/HRP peroxidase conjugate (Bio-Rad) as the secondary antibody. Bands were detected using a chemiluminescent substrate (SuperSignal West Femto; Pierce) and photographed using a digital camera (Alpha Innotech Corp.). As a control for loading and transfer, parallel blots were stained for total protein with 0.1% Ponceau S, 1% acetic acid and scanned using an Epson Perfection 636 scanner.

**Integrin β3 Antisense Inhibits Capillary Tube Formation**—A modification of the method of Nehls and Drenckhahn (43) was used to measure endothelial cell capillary tube formation in a three-dimensional fibrin-based matrix. HMEC-1 cells were grown to confluence on Cytoxid-3 microcarriers (Amersham Pharmacia Biotech) for 2–3 days in spinner flasks at 37 °C with 5% CO2. Confluent HMEC-1 cell-coated microcarriers were rinsed three times and resuspended in tube formation assay medium: Dulbecco's modified Eagle's medium, 20% fetal bovine serum, 2 mM L-glutamine, 200 kallikrein-inactivating units/ml aprotinin (Bayer). HMEC-1 cell-coated microcarriers were added to sterile-filtered solutions containing 1.5 mg/ml fibrinogen in PBS with 200 kallikrein-inactivating units/ml aprotinin and 30 ng/ml bFGF (R & D Systems). The concentration of bFGF was the same as that used by Nehls and Drenckhahn (43) for stimulating endothelial cell capillary tube formation. Abciximab (obtained from Dr. Mark Kozak, Penn State University, Hershey, PA) was dissolved in assay medium, and 50 μl was added on top of the clot and incubated at 37 °C with 5% CO2 for 1 h. An additional 50 μl of tube formation assay medium was then added, and plates were incubated at 37 °C with 5% CO2 for 2–5 days. Cell nuclei were stained for 2 h with 50 μg/ml bisbenzimide (Sigma). After inverting the plate, cell sprouts, defined as projections containing a minimum of three nuclei (43), were counted using fluorescence microscopy. The mean number of sprouts per microcarrier was determined in triplicate, and each assay was performed a minimum of three times. Photomicrographs were taken using an Olympus B-Max 50 epifluorescence microscope with a Polfilter cooled charge-coupled device camera interfaced with a Scion LG3 framestore board mounted in a Macintosh Centris 650 computer and running Adobe Photoshop.

**Transmission Electron Microscopy**—Transmission electron microscopy was performed using a modification of the method of Karnovsky (44). Capillary tube formation assays were performed as described up to the point of bisbenzimide staining. HMEC-1 cells were then rinsed three times with 0.1 M sodium cacodylate, pH 7.3, and fixed in 4% paraformaldehyde, 2.5% glutaraldehyde, 0.5 mM CaCl2, 0.1 M sodium cacodylate, pH 7.3 for 2 h at 4 °C. HMEC-1 cells were rinsed three times at 5-min intervals in sodium cacodylate, pH 7.3, and postfixed in 1% osmium tetroxide, 1.5% potassium ferrocyanide, 0.1 M sodium cacodylate, pH 7.3, at 4 °C overnight. Specimens were rinsed three times at 5-min intervals with 0.1 M sodium cacodylate, pH 7.3, dehydrated in a graded ethanol series, and embedded in 49% EM bed-812, 32% dodecyl nitril succinic anhydride, 20% nadic methyl anhydride, 1.4% 2,4,6-tri-
**β3 Integrin cDNA Antisense Fragments**

| Fragment Size | Percentage |
|---------------|------------|
| 130 bp        | 59.7%      |
| 240 bp        | 21.2%      |
| 419 bp        | 11.7%      |
| 426 bp        | 6.4%       |

**Results**

**β3 Antisense Constructs**—To down-regulate αvβ3 expression, the β3 integrin subunit mRNA was targeted with RNA antisense. The region of a cDNA that yields effective antisense RNA cannot be predicted with certainty; therefore, four different β3 antisense constructs were tested from different regions of the β3 cDNA. The sequences selected for antisense targeting were designed to avoid regions of high homology among β subunits (45) (Fig. 1, hatched bars), with the exception of β3 419. A 130-bp β3 antisense cDNA fragment (β3 130 antisense) from nucleotides 1–130 preceded the first homologous domain and included one intron/exon boundary and the translational start site; a 240-bp β3 antisense cDNA fragment (β3 240 antisense) from nucleotides 180–419 between the first and second homologous domains included two intron/exon boundaries; a 419-bp β3 antisense cDNA fragment (β3 419 antisense) from nucleotides 1–419 included the same region as β3 130 antisense, but extended through the first homologous domain and ended before the second domain, and contained three intron/exon boundaries; and a 426-bp β3 antisense cDNA fragment (β3 426 antisense) from nucleotides 1068–1493 between the seventh and eighth homologous domains included two intron/exon boundaries. These sequences were amplified using PCR and inserted in a sense or antisense orientation into the mammalian expression vector pCEP-4/hygroycin.

The HMEC-1 human microvascular endothelial cell line was chosen for transfection with these constructs rather than primary endothelial cells, since it is difficult, if not impossible, to isolate stable transfectants from primary microvascular endothelial cells in culture. Transfectants that are isolated from such primary cells have often undergone phenotypic changes and have a limited lifespan prior to senescence. In contrast, the HMEC-1 cell line is a diploid cell line that retains the characteristics of microvascular endothelial cells during prolonged culture in vitro and does not differentiate (46). β3 sense and antisense constructs, as well as a full-length β3/pCEP-4 construct and the pCEP-4 vector alone were stably transfected into HMEC-1 cells. Eight to ten individual clones were isolated for each construct, since vector copy number can alter gene expression in any particular clone.

**β3 240 Antisense Expression Specifically Inhibits αvβ3 Levels**—The relative levels of αvβ3 on transfected and nontransfected HMEC-1 cell clones were determined using flow cytometry. β3 130 antisense and β3 419 antisense transfectants had reductions in αvβ3 levels of 28–62% and up to 33%, respectively (data not shown). However, these transfectants also had comparable decreases in β3 levels that could complicate the analysis of the specific role of αvβ3 and therefore were not characterized further. In contrast, β3 240 antisense transfectants had reductions in αvβ3 levels of 0–49% with no concomitant changes in β3 levels and showed no significant changes in αvβ5 levels (Table I). β3 426 antisense transfectants had somewhat smaller reductions in αvβ3 levels than the β3 240 antisense transfectants, 2–29% (Table I), and were therefore not characterized further. HMEC-1 cells transfected with pCEP-4 alone or the β3 240 fragment in the sense orientation had levels of αvβ3, β3, and αvβ5 that were comparable with nontransfected HMEC-1 cells (Table I). HMEC-1 cells transfected with full-length β3 in the sense orientation also showed no significant changes in αvβ3 levels (Table I). Cells transfected with the β3 240 fragment in the sense or antisense orientation expressed the appropriate sense or antisense RNA, as detected by semiquantitative RT-PCR, whereas these transcripts were undetectable in wild-type HMEC-1 cells (Fig. 2). Although the primers will also amplify the endogenous mRNA for β3, bands from the endogenous mRNA were not visible in wild-type HMEC-1 cells under the conditions used to amplify the sense and antisense fragments, consistent with the higher expression levels of the fragments from the viral promoters in the pCEP-4 expression vector.

Western blot analysis of the β3 antisense transfectants showed similar amounts of αv expressed compared with the

**Table I**

| HMEC-1 Transfectant | αvβ3 level | β3 level | αvβ5 level |
|---------------------|------------|----------|------------|
| B pCEP4             | 105 ± 4    | 99 ± 2   | 119 ± 12   |
| E pCEP4             | 102 ± 9    | 97 ± 6   | 113 ± 5    |
| L pCEP4             | 96 ± 6     | 116 ± 6  | 108 ± 10   |
| 2 pCEP4             | 100 ± 5    | 103 ± 2  | 101 ± 4    |
| 3 pCEP4             | 93 ± 6     | 103 ± 6  |            |
| A β3 240 sense      | 101 ± 7    | 105 ± 7  | 103 ± 5    |
| 1 β3 240 sense      | 103 ± 6    | 104 ± 1  | 99 ± 13    |
| 2 β3 240 sense      | 106 ± 2    | 92 ± 1   | 100 ± 9    |
| 3 β3 240 sense      | 97 ± 4     | 105 ± 1  | 107 ± 2    |
| 9 β3 240 sense      | 99 ± 3     | 111 ± 9  | 113 ± 12   |
| W β3 240 antisense  | 69 ± 6     | 103 ± 3  | 98 ± 9     |
| 2 β3 240 antisense  | 60 ± 4     | 105 ± 7  | 93 ± 6     |
| 10 β3 240 antisense | 51 ± 4     | 104 ± 4  | 103 ± 8    |
| 17 β3 240 antisense | 100 ± 4    | 100 ± 3  | 95 ± 8     |
| 20 β3 240 antisense | 76 ± 5     | 104 ± 1  | 102 ± 4    |
| 6 β3 426 sense      | 100 ± 6    |          |            |
| 8 β3 426 sense      | 100 ± 7    |          |            |
| A β3 426 antisense  | 98 ± 2     |          |            |
| C β3 426 antisense  | 92 ± 4     |          |            |
| 1 β3 426 antisense  | 85 ± 5     |          |            |
| 3 β3 426 antisense  | 71 ± 7     |          |            |
| 1 β3 full-length    | 98 ± 6     |          |            |
| 2 β3 full-length    | 104 ± 3    |          |            |
| 3 β3 full-length    | 100 ± 6    |          |            |
| 8 β3 full-length    | 99 ± 2     | 107 ± 8  | 104 ± 2    |
| 17 β3 full-length   | 101 ± 4    |          |            |
| 19 β3 full-length   | 105 ± 5    |          |            |
parental HMEC-1 cells (Fig. 3), whereas \( \beta_3 \) expression was markedly reduced. Scanning densitometry of the \( \alpha_v \) and \( \beta_3 \) bands in Fig. 3 showed only a 2% difference in \( \alpha_v \) band intensity, compared with a 40% reduction in \( \beta_3 \) band intensity for the \( \beta_3 \) 240 antisense transfectant. These results demonstrate that the \( \beta_3 \) 240 antisense transfectant had decreased levels of \( \beta_3 \) expression, but \( \alpha_v \) expression was not affected. Interestingly, no compensatory increases in \( \alpha_v \beta_3 \) levels were seen when \( \alpha_v \beta_3 \) levels were decreased in the \( \beta_3 \) 240 antisense transfectants. This is in contrast to the compensatory changes seen in individuals with a certain type of Glanzmann thrombasthenia. In individuals with defects in \( \alpha_\text{IIb} \beta_3 \) that result in decreased \( \alpha_\text{IIb} \beta_3 \) levels on platelets, a compensatory elevation in \( \alpha_\text{IIb} \beta_3 \) levels can occur due to pairing of the excess \( \beta_3 \) subunits with \( \alpha_v \) (16). However, antisense inhibition of \( \beta_3 \) expression did not cause compensatory increases in \( \alpha_v \beta_3 \) levels due to pairing of the excess \( \alpha_v \) subunits with \( \beta_3 \) in the transfected cells. It is not clear if the excess \( \alpha_v \) subunits are paired with \( \beta_3 \) or retained intracellularly.

**HMEC-1 Cells Form Lumen-containing Capillary-like Structures in a Three-dimensional Fibrin Matrix**—Although the inhibition of \( \alpha_v \beta_3 \) expression on \( \beta_3 \) 240 antisense transfectants was not complete, the reductions in \( \alpha_v \beta_3 \) appeared specific, and the cells were therefore tested for their ability to form capillary tubes. Angiogenesis is a multifactorial process that has been divided into at least six distinct steps: 1) proteolytic digestion of extracellular matrix, 2) migration of endothelial cells, 3) proliferation of these cells, 4) extracellular matrix production, 5) vascular tube formation, and 6) Anastomosis of newly formed channels resulting in a patent neovessel (47). Assays have been developed to study each of these steps in vitro, thereby providing a reductionist approach to the complex process of angiogenesis. In particular, endothelial cell tube formation assays have been used to identify critical modulators of this process (48). Because the effects of angiogenesis modulators in two-dimensional tube formation assays do not always correlate with their effects in vivo (47), three-dimensional capillary tube formation assays have been developed. These latter assays appear to more faithfully recapitulate the process of angiogenesis (49).

We modified the well characterized three-dimensional capillary tube formation assay developed by Nehls and Drenckhahn (43) to use HMEC-1 cells that were grown to confluence on microcarriers and embedded in a fibrin matrix in the presence of 30 ng/ml bFGF. HMEC-1 cells were cultured in the three-dimensional fibrin matrix for 2–4 days at 37 °C. To ensure that this in vitro capillary tube formation assay responded to authentic angiogenic modulators, several parameters were examined. HMEC-1 cells without thrombin added to clot the fibrinogen migrated randomly from the microcarrier beads onto the well and did not form capillary-like structures (data not shown). HMEC-1 cells embedded in the fibrin matrix in the absence of an angiogenic stimulus migrated from the microcarriers into the fibrin in a somewhat more organized fashion but formed few distinguishable capillary-like sprouts (data not shown). In contrast, HMEC-1 cells cultured in the presence of all the assay components formed capillary-like sprouts, similar to those seen when large vessel bovine pulmonary artery endothelial cells were used in this assay (43). HMEC-1 cells migrated from the microcarrier into the fibrin matrix and formed multicellular lumen-containing capillary-like structures (Fig. 4A) similar to those formed with bovine endothelial cells (43). Cell/cell junctions were clearly visible between HMEC-1 cells forming capillary-like structures, and the subcellular structures appeared normal (Fig. 4B). Therefore, HMEC-1 cells appeared to undergo capillary tube formation in this in vitro assay similarly to large vessel bovine pulmonary artery endothelial cells (43).

**Antibodies against \( \alpha_v \beta_3 \) Inhibit Microvascular Endothelial Cell Capillary Tube Formation in a Fibrin Matrix**—To test that microvascular endothelial cell capillary tube formation was dependent on integrin \( \alpha_v \beta_3 \) in this assay, HMEC-1 cells on microcarriers were embedded in a fibrin matrix in the presence of monoclonal antibodies directed against particular integrins. The monoclonal antibodies used were abciximab, a chimeric Fab fragment of the monoclonal antibody 7E3 (50), LM609, directed against \( \alpha_\text{IIb} \beta_3 \) (25), and as a negative control, JB1a directed against \( \beta_3 \) integrins (37). Although abciximab was originally developed as an antagonist of platelet \( \alpha_{\text{IIb}} \beta_{3} \), recent studies demonstrated that abciximab binds with comparable affinity to \( \alpha_v \beta_3 \) (51). After 2–4 days of culture, HMEC-1 nuclei were stained with bisbenzimide and photographed to quantify the effect on capillary tube formation. Capillary tube formation was quantitated as the number of sprouts per microcarrier bead, with a sprout defined as a minimum of three interconnected cells (43). Control HMEC-1 cells stimulated...
Integrin $\beta_3$ Antisense Inhibits Capillary Tube Formation

DISCUSSION

These results demonstrate that antisense-mediated down-regulation of $\alpha_v\beta_3$ expression in microvascular endothelial cells inhibits capillary tube formation in fibrin. Furthermore, the extent of $\alpha_v\beta_3$ down-regulation correlates with the extent of tube formation inhibition. This inhibition is matrix-specific, since tube formation is not inhibited in Matrigel. Although these results were obtained using a cell line, this cell line has a normal diploid genetic complement and retains many characteristics of normal human microvascular endothelial cells (33), seen when the cells were treated with 5 $\mu$g/ml of the documented angiogenesis inhibitor LM609 (Fig. 5C). In contrast, 5 $\mu$g/ml of JB1a had no effect on endothelial capillary tube formation (Fig. 5D). Both abciximab and LM609 inhibited HMEC-1 capillary tube formation in a dose-dependent fashion from 29 to 80% and from 63 to 89%, respectively, with 0.01 to 5 $\mu$g/ml, while JB1a had no effect (data not shown). LM609 antibody was more effective than abciximab at inhibiting capillary tube formation at concentrations of 0.01–1 $\mu$g/ml, but at 5 $\mu$g/ml, abciximab and LM609 inhibited fibrin-based capillary tube formation to a similar extent (data not shown). The differences in dose response may reflect the fact that abciximab is a Fab fragment, whereas LM609 is intact IgG, or they may reflect a difference in antibody affinity for $\alpha_v\beta_3$ (51).

$\beta_3$ 240 Antisense Expression Inhibits Microvascular Endothelial Cell Capillary Tube Formation in a Fibrin Matrix—$\beta_3$ 240 antisense-transfected HMEC-1 cells were tested in the capillary tube formation assay to examine the effect of reducing $\alpha_v\beta_3$ levels. Several independent clones were analyzed for each type of transfected HMEC-1 cell. pCEP-4-transfected HMEC-1 cells and $\beta_3$ 240 sense-transfected HMEC-1 cells (Fig. 6, B and C, respectively; Table II) sprouted to a similar extent as nontransfected HMEC-1 cells (Fig. 6A and Table II). All full-length $\beta_3$ sense clones sprouted similarly to nontransfected HMEC-1 cells (data not shown). In contrast, all of the $\beta_3$ 240 antisense transfectants with reduced $\alpha_v\beta_3$ levels consistently showed reduced sprouting (Fig. 6D). The only $\beta_3$ 240 antisense transfectant that showed no change in $\alpha_v\beta_3$ level, clone 17, also showed no change in sprouting (Table II). The number of sprouts/microcarrier increased in a dose-dependent manner with the level of $\alpha_v\beta_3$ in fibrin-based tube formation assays (Fig. 7). Although the $\alpha_v\beta_3$ level on the $\beta_3$ 240 antisense transfectants was reduced by only 24–49%, tube formation in fibrin was inhibited by 21–76%. The maximal inhibition of tube formation seen for the $\beta_3$ 240 antisense transfectants was comparable with the inhibition seen with maximal doses of abciximab and LM609 (Fig. 5, B and C). Although the $\alpha_v\beta_3$ down-regulation correlates with the extent of $\alpha_v\beta_3$ level on the $\beta_3$ 240 antisense transfectants was comparable with the inhibition seen with maximal doses of abciximab and LM609 (Fig. 5, B and C).

$\beta_3$ 240 Antisense Expression Does Not Inhibit Microvascular Endothelial Cell Capillary Tube Formation in a Matrigel Matrix—To determine if the effect of $\beta_3$ antisense expression on tube formation was specific to fibrin matrices, a Matrigel-based tube formation assay was performed. Whereas endothelial cell interactions with fibrinogen and fibrin are largely $\alpha_v\beta_3$-dependent (25, 52–54), capillary-like structures formed in laminin and collagen IV matrices such as Matrigel are largely dependent on interactions with endothelial cell laminin and collagen receptors (9–11) such as $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_1$, and $\alpha_v\beta_1$ (laminin receptor). Capillary formation by HMEC-1 cells in Matrigel was inhibited by a monoclonal antibody directed against $\beta_3$ integrins, JB1a (Fig. 8, J–L), whereas no inhibition was seen with abciximab (Fig. 8, D–F) or LM609 (Fig. 8, G–I). HMEC-1 cells transfected with pCEP-4 alone, $\beta_3$ 240 sense, or $\beta_3$ 240 antisense all formed capillary-like structures in Matrigel similar to nontransfected HMEC-1 cells (data not shown). The Matrigel tube formation assay shows that the $\beta_3$ antisense effect is matrix-specific.

Fig. 4. HMEC-1 cells form lumen-containing capillary-like sprouts with cell/cell junctions in a fibrin matrix. A three-dimensional fibrin-based capillary tube formation assay was performed using bFGF-stimulated HMEC-1 cells. On day 2, the HMEC-1 cells were fixed, dehydrated in a graded ethanol series, and embedded for sectioning. Thin sections were cut and stained, and photographs were taken. A, HMEC-1 cells have formed lumen-containing capillary-like structures. Magnification, $\times$ 5784. B, cell/cell junctions between HMEC-1 cells forming the capillary-like structures. Magnification, $\times$ 44,385. MC, microcarrier; L, lumen; N, nucleus; J, junction.

with bFGF sprouted and migrated into the fibrin matrix (Fig. 5A). In contrast, cells stimulated with bFGF but treated with 5 $\mu$g/ml abciximab showed significant inhibition of capillary tube formation (Fig. 5B). The inhibition was comparable with that
suggesting that the results obtained may be extrapolated to primary cells. The assay may also prove useful as a rapid screening method for pro- and antiangiogenic agents. In addition, a \( \beta_3 \) down-regulation in these studies did not involve the use of pharmacologic agents with pleiotypic effects. Moreover, evidence for a true antisense effect was demonstrated in trans-

**FIG. 5.** Capillary tube formation by HMEC-1 cells in fibrin is inhibited by antibodies against \( \alpha_\beta \). A three-dimensional fibrin-based capillary tube formation assay was performed using bFGF-stimulated HMEC-1 cells. On day 2, the nuclei were stained with bisbenzimide and photographed. A, control HMEC-1 cells showed numerous capillary-like sprouts. HMEC-1 cells incubated with 5 \( \mu \)g/ml abximab (B) or 5 \( \mu \)g/ml anti-\( \alpha_\beta_3 \) LM609 (C) showed a significant decrease in the number of sprouts per microcarrier. D, HMEC-1 cells incubated with 5 \( \mu \)g/ml anti-\( \beta_1 \) JB1a) showed similar capillary tube formation to control HMEC-1 cells. Bar, 100 \( \mu \)m.

**FIG. 6.** \( \beta_3 \) 240 antisense expression inhibits capillary tube formation in fibrin. A three-dimensional fibrin-based tube formation assay was performed using bFGF-stimulated HMEC-1 cells. On day 3, nuclei were stained with bisbenzimide and photographed. Nontransfected HMEC-1 cells (A), pCEP-4 transfectants (B), and \( \beta_3 \) 240 sense transfectants (C) showed numerous capillary-like sprouts, but \( \beta_3 \) 240 antisense transfectants showed a significant decrease in sprouting (D).

**TABLE II**

Capillary tube formation in fibrin of HMEC-1 transfectants

| HMEC-1 transfectant | Sprouts/microcarrier |
|---------------------|----------------------|
| B pCEP4             | 94 ± 4               |
| E pCEP4             | 105 ± 10             |
| L pCEP4             | 92 ± 15              |
| A \( \beta_3 \) 240 sense | 99 ± 9               |
| 1 \( \beta_3 \) 240 sense | 96 ± 8               |
| 3 \( \beta_3 \) 240 sense | 95 ± 4               |
| W \( \beta_3 \) 240 antisense | 79 ± 2               |
| 2 \( \beta_3 \) 240 antisense | 39 ± 4               |
| 10 \( \beta_3 \) 240 antisense | 24 ± 5               |
| 17 \( \beta_3 \) 240 antisense | 10 ± 7               |

**Fig. 7.** \( \alpha_\beta_3 \) expression correlates with capillary tube formation in fibrin. Flow cytometry was conducted using primary antibody anti-\( \alpha_\beta_3 \) LM609. A three-dimensional fibrin-based tube formation assay was conducted using bFGF-stimulated HMEC-1 cells. On day 3, nuclei were stained with bisbenzimide, and sprouting of nontransfected and transfected HMEC-1 cells was quantitated. The number of sprouts/microcarrier for transfected clones was normalized to the number of sprouts/microcarrier for nontransfected HMEC-1 cells. The data shown are one representative of three such experiments, in which each point represents the mean of triplicate determinations ± S.D.
Fig. 8. Capillary tube formation by HMEC-1 cells in Matrigel is inhibited by antibodies against \( \beta_3 \) integrins but not \( \beta_1 \) integrins. Matrigel-based capillary tube formation assays were conducted, and representative fields from one of three experiments are shown at \( \times \) 100 magnification. Control HMEC-1 cells (A–C) formed capillary-like structures in Matrigel. HMEC-1 cells incubated with 5, 20, or 50 \( \mu \)g/ml abciximab (D–F) or 5, 20, or 50 \( \mu \)g/ml anti-\( \alpha_\text{v} \beta_3 \) (LM609) (G–I) formed capillary-like structures on Matrigel similar to control HMEC-1 cells, but anti-\( \beta_3 \) (JB1a) at 5, 20, or 50 \( \mu \)g/ml (J–L) significantly reduced capillary-like structure formation in a dose-dependent manner.

RNA antisense to integrin \( \beta_3 \) may provide a novel approach to specific \( \alpha_\text{v} \beta_3 \) antagonism that could be used therapeutically for angiogenesis-dependent pathologies. Li et al. (58) have used full-length \( \beta_3 \) antisense to down-regulate \( \alpha_\text{v} \beta_3 \) expression in tumors and shown that cell motility and basement membrane invasion was reduced significantly. However, the effect of this antisense construct on the expression of related integrins such as \( \beta_1 \) was not characterized. Our results provide proof of the concept that an antisense fragment to integrin \( \beta_3 \) subunit can be used to specifically reduce \( \alpha_\text{v} \beta_3 \) levels and inhibit microvascular endothelial cell capillary tube formation in fibrin and provide an impetus for further investigations of \( \beta_3 \) antisense as an angiogenesis antagonist. In particular, direct delivery of synthetic oligodeoxynucleotides or virus-mediated infection of endothelial cells (59) to express the antisense RNA endogenously may provide methods to inhibit angiogenesis in vivo.

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