

Regulation of In Vitro Capillary Tube Formation by Anti-Integrin Antibodies

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Abstract. Human endothelial cells are induced to form an anastomosing network of capillary tubes on a gel of collagen I in the presence of PMA. We show here that the addition of mAbs, AK7, or RMAC11 directed to the α chain of the major collagen receptor on endothelial cells, the integrin $\alpha_2\beta_1$, enhance the number, length, and width of capillary tubes formed by endothelial cells derived from umbilical vein or neonatal foreskins. The anti- $\alpha_2\beta_1$ antibodies maintained the endothelial cells in a rounded morphology and inhibited both their attachment to and proliferation on collagen but not on fibronectin, laminin, or gelatin matrices. Furthermore, RMAC11 promoted tube formation in collagen gels of increased density which in the absence of RMAC11 did not allow tube formation. Neither RMAC11 or AK7 enhanced capillary formation in the absence of PMA. Lumen structure and size were also altered by antibody RMAC11. In the absence of antibody the majority of lumina were formed intra-

cellularly from single cells, but in the presence of RMAC11, multiple cells were involved and the lumen size was correspondingly increased. Endothelial cells were also induced to undergo capillary formation in fibrin gels after PMA stimulation. The addition of anti- $\alpha_v\beta_3$ antibodies promoted tube formation in fibrin gels and inhibited EC adhesion to and proliferation on a fibrinogen matrix. The enhancement of capillary formation by the anti-integrin antibodies was matrix specific; that is, anti- $\alpha_v\beta_3$ antibodies only enhanced tube formation on fibrin gels and not on collagen gels while anti- $\alpha_2\beta_1$ antibodies only enhanced tubes on collagen and not on fibrin gels. Thus we postulate that changes in the adhesive nature of endothelial cells for their extracellular matrix can profoundly effect their function. Anti-integrin antibodies which inhibit cell-matrix interactions convert endothelial cells from a proliferative phenotype towards differentiation which results in enhanced capillary tube formation.

NEW blood vessel formation is an essential event in embryogenesis and wound healing. However, little is known about the steps which induce flat, static endothelial cells (EC)¹ to undergo differentiation leading to a new capillary bed. Recently, a number of in vitro assays have been established which are thought to mimic angiogenesis and these have provided insight into possible mechanisms. EC initially undergo a spatial reorientation towards the angiogenic stimulus, invade and disrupt the extracellular matrix (ECM) by production of matrix degrading enzymes such

as plasminogen activator and collagenase and undergo tube formation and extension via EC proliferation (for review see Folkman and Handenschild, 1980; Folkman, 1986). Thus, measurement of EC realignment, enzyme secretion, and cell proliferation are taken as indicators of EC differentiation and tube formation. Using such assays, it is clear that a number of factors including basic fibroblast growth factor, TGF- β , and tumor necrosis factor (Folkman and Klagsbrun, 1987) (termed angiogenic factors), can stimulate angiogenesis. In addition to the requirement for an angiogenic factor, in vitro capillary formation is also dependent on the correct matrix. Endothelial cells, plated on a two-dimensional matrix of ECM proteins or on plastic, form capillary tubes slowly (2–3 wk) (Kubota et al., 1988; Iruela-Arispe et al., 1991). However, when plated onto a gel of the ECM proteins, tubes form within 24 h (Kubota et al., 1988). Isolated components of the ECM, such as collagen or fibrinogen, when gelled are also able to induce the formation of capillary tubes (Monte-

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1. *Abbreviations used in this paper:* EC, endothelial cells; ECGS, endothelial cell growth supplement; ECM, extracellular matrix; HUVEC, human umbilical vein endothelial cell; MVEC, microvessel EC; uPA, urokinase plasminogen activator.

sano et al., 1985). In collagen and fibrin gels the process of capillary formation is enhanced by the activation of the EC with the tumor promoter, PMA (Montesano and Orci, 1985; Montesano et al., 1987). Thus, even in the presence of an angiogenic factor, and a permissive milieu, an additional signal is required and this can be provided by PMA.

Cell surface molecules mediating adhesion to either neighboring cells or to substrates are likely to play a key role in angiogenesis. The integrins are a family of cell surface molecules which mediate the attachment of cells to the ECM and to other cells. At least 19 different cell-surface $\alpha\beta$ heterodimers have been identified, some of which mediate adhesion to ECM proteins such as laminin, collagen, fibrinogen, and fibronectin (Ruoslahti and Pierschbacher, 1987; Hynes, 1987; Albelda and Buck, 1990). EC express five of the six β_1 integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$) although there is some heterogeneity in the level of expression depending on the source of the EC (Defilippi et al., 1991a). The major collagen receptor on EC is $\alpha_2\beta_1$ (Albelda et al., 1989; Languino et al., 1989) which can also mediate binding to laminin (Kramer et al., 1990). EC also express $\alpha_v\beta_3$ which mediates adhesion to fibrinogen (Albelda et al., 1989; Cheresh and Spiro, 1987) as well as laminin (Kramer et al., 1990) and vitronectin (Cheresh and Spiro, 1987).

We describe here for the first time that anti-integrin antibodies directed to the receptors mediating attachment to the tube-permissive matrices of collagen I and fibrin enhance capillary formation as measured by tube number, length, and thickness. Anti-integrin antibodies enhance tube thickness by increasing the number of cells involved in lumen formation. These results suggest that capillary tube formation is dependent on the interaction of EC with the ECM and that restriction of specific cell-matrix interactions can enhance the extent of capillary formation.

Materials and Methods

Endothelial Cells

Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase treatment as described (Wall et al., 1978). The cells were cultured in 25 cm² gelatin (Eastman Kodak Co., Rochester, NY)-coated flasks (Costar Corp., Cambridge, MA) in M199 with Earles Salts, 20 mM Hepes, 20% FCS (Cytosystems, Sydney), sodium bicarbonate, 2 mM glutamine, nonessential amino acids, sodium pyruvate, fungizone, penicillin, and gentamycin (HUVEC medium). Cells were grown at 37°C, 5% CO₂. Within 2–4 d the HUVEC formed a confluent monolayer and were then harvested by trypsin-EDTA treatment and transferred to a 75-cm² gelatin-coated flask. 50 μ g/ml of endothelial cell growth supplement (ECGS, Collaborative Research, Bedford, MA) and 50 μ g/ml of heparin (Sigma Chem. Co., St. Louis, MO) were added. Cells were passaged (1:2 split) every 3–4 d, and were used between passage 2 and 6. Microvessel EC (MVEC) were prepared from neonatal foreskins according to the method of Marks et al. (1985). Cells were frozen in liquid nitrogen at $1-2 \times 10^6$ /vial at passage 2–6 and thawed as required. Cells were grown to confluence before use. Medium for growth and maintenance of these EC was M199 with Earles Salts, 25 mM Hepes, 50% human serum, sodium bicarbonate, 2 mM glutamine, fungizone, penicillin, streptomycin, 3.3×10^{-4} M cAMP, ECGS (50 μ g/ml), and heparin (50 μ g/ml).

Collagen Gel Capillary Assay

Bovine Type I collagen (Celtrix Laboratories, Palo Alto, CA) gel was prepared by simultaneously raising the pH and the ionic strength of a collagen solution, using a modification (Greenburg and Hay, 1982) of the original

method described by Elsdale and Bard (1972). Seven volumes of ice cold collagen solution (3 mg/ml) was mixed with 1 vol of 10 \times concentrated PBS, pH 7.4, and 2 vol of sodium bicarbonate (11.76 mg/ml) on ice. One hundred μ l of the mixture was aliquoted into 96 well flat-bottomed microtiter trays (Nunc, Roskilde, Denmark) and allowed to gel for 10–20 min at 37°C. For more rigid collagen gels, the mixture was allowed to gel for at least 1 h at 37°C. After gel formation, EC, which were removed from confluent monolayers by trypsin treatment, were plated down onto the gel at a concentration of 6.4×10^4 cells/160 μ l in HUVEC or MVEC medium with ECGS and heparin. As indicated 20 ng/ml PMA (Sigma Chem. Co.) was added to some wells. In some assays, as indicated, cells together with PMA were resuspended in the collagen before gelling (modification of method by Madri et al., 1988).

Fibrin Gel Capillary Assay

Three dimensional fibrin gels were prepared as previously described by Montesano et al. (1985). One hundred μ l of plasminogen free fibrinogen, 3 mg/ml in PBS (Sigma Chem. Co.) was placed into 96 well flat-bottomed microtiter wells (Nunc) and clotted by the addition of 2 μ l of 1 U/ml thrombin (Parke Davis Pty Ltd., Adelaide, Australia) in PBS. The mixture was allowed to gel for \sim 2 min at 37°C before addition of EC. Cell numbers and conditions were the same as that described for the collagen gel assay.

Quantification of Capillary Formation

Tube formation was assessed at several different focal plains through the gel. The extent of capillary tube formation was judged in relation to the amount of EC monolayer and to the number, width, and length of the tubes formed. Based on these criteria values from + to ++++ were assigned. Tube formation was also quantified from high power photographs. At least two photographs from random fields from each microtiter well (duplicate wells were set up for each group) were taken. Areas of the well were avoided where the meniscus gave a distortion of the optics. We defined tubes as straight cellular extensions joining two cell masses or branch points. The minimum width of tubes was measured and is given as the width. From the photographs, counts were made of the number, length, and width of tubes.

Proliferation Assay

The mitogenic response of EC to different stimuli was measured using a spectrophotometric assay (Oliver et al., 1989). Cells (5×10^3) were plated onto matrix-coated flat-bottomed microtiter trays (Nunc), 150 μ l/well in HUVEC medium either with or without ECGS, and heparin. After 3 d, medium was removed and the cells fixed in 10% formal saline for 30 min. One hundred μ l of methylene blue (1% wt/vol, in 0.01 M borate buffer, pH 8.5) was then added to each well, incubated for 30 min, the stain flicked off, and the cells washed 3–4 times in borate buffer. The dye was released by addition of ethanol, 0.1 M HCl solution (1:1) with a brief shaking. The optical density at 630 nm (OD₆₃₀ nm) was then determined. The percentage proliferation was calculated based on the OD₆₃₀ of the no antibody control group normalized to 100%. Experiments showed that there was a linear relationship between the OD₆₃₀ nm and the increase in cell number and that as low as 10^3 cells/well could be detected. Similar results were obtained using the methylene blue assay and the uptake of [³H]thymidine (data not shown).

Cell Attachment Assays

Microtiter plates were coated with either collagen I (50 μ g/ml), gelatin (1%), or fibronectin (100 μ g/ml) for 30 min at room temperature. Fibrin-coated microtiter wells were formed by thrombin treatment (1 U/ml) of fibrinogen which had been previously added to wells. Wells were washed twice with PBS and 5×10^3 cells were added per well in 50 μ l of HUVEC medium without FCS. Plates were incubated at 37°C for the indicated times and cell attachment quantitated using the methylene blue assay (as outline above).

Collagenase Assay

Measurement of active collagenase was performed according to the method of Nethery et al. (1986). Microtiter wells were coated with 0.7 mg/ml collagen I solution (Collaborative Research Inc.), rinsed in water, and air dried. Cell supernatants were treated with 0.5 mg/ml trypsin at 37°C followed by treatment with soybean trypsin inhibitor (Sigma Chem. Co.) at 5 mg/ml.

Collagenase samples (CLS-1 Worthington Biochem. Corp., Freehold, NJ) were prepared at concentrations from 1 to 1,000 $\mu\text{g/ml}$ in assay buffer (50 mM tris-HCl, 100 mM NaCl 10 mM CaCl_2 , pH 7.5) and were used to generate a standard curve. One hundred μl of samples and standards were then added to the collagen-coated microtiter wells and incubated for 16 h at 37°C. The wells were washed and stained using 0.25% Coomassie blue R-250 (Biorad Labs., NSW, Australia) for 25 min at room temperature. The stain was removed, wells washed in water, dried, and the absorbance at 590 nm determined. The level of collagenase present in the sample was inversely proportional to the optical density reading at 590 nm. The level of detection was 10 ng/ml.

Monoclonal Antibodies

RMAC11 binds the α chain of the $\alpha_2\beta_1$ integrin complex on endothelial cells and fibroblasts and QE2E5 binds the β_1 chain (O'Connell et al., 1991). Preclearing and immunoprecipitation experiments show that AK7 also recognizes the α chain of the $\alpha_2\beta_1$ complex (Mazurov et al., 1991). RMAC11 (IgG2a), QE2.E5 (IgG2b), and AK7 (IgG1) were purified from ascites fluid using a mAb Trap G Sepharose column (Pharmacia LKB, NSW, Australia). Fab₂ fragments of RMAC11 were prepared by pepsin digestion and Fab fragments by papain digestion of the IgG according to the method of Harlow and Lane (1988). P4C10, an IgG₁ antibody directed to the β_1 integrin chain (Carter et al., 1990) was kindly provided by Dr. W. G. Carter, Fred Hutchinson Cancer Research Center, Seattle, WA. Another antibody, 61.2C4, has been shown to be directed to the β_1 chain (Gamble, J. R., and M. A. Vadas, unpublished observations). LM609 and 13C2, both anti- $\alpha_3\beta_3$ antibodies, were kindly provided by Dr. David Chersesh, Scripps, La Jolla, CA and Dr. Michael Horton, Department of Haematology, St. Bartholomew's Hospital, London, respectively.

Analysis by Flow Cytometry

EC were plated on collagen gels either in the presence or absence of PMA. 24 h later, cells either as tubes or as a monolayer were extracted by treatment of the gels for 30 min in 2 mg/ml collagenase at 37°C. Cells were then trypsin-treated for 5 min to obtain single cell suspensions. Cells were stained with the appropriate antibodies for 30 min at 37°C followed by a rabbit anti-mouse-FITC antibody for 30 min at 4°C. Cells were washed three times in PBS and diluted in fixative (2% glucose, 5 mM sodium azide, 1% formaldehyde) before analysis on an Epics Profile Analyzer. Ten thousand cells were analyzed.

Microscopy

At 24 h after stimulation with PMA or PMA and RMAC11, culture medium was removed from the culture wells and replaced with 2% paraformaldehyde, 2.5% glutaraldehyde in sterile PBS (pH 7.4). Cultures were fixed in this solution for 12 h at room temperature (20°C). After 12 h, fixative was washed out with PBS, at least 10 changes every 10 min, and the cultures postfixed for an additional 12 h in 1% osmium tetroxide in PBS. The osmium tetroxide was washed from the cultures, at least 10 changes every 10 min. Cultures were then dehydrated for three 20-min periods in each of a graded series of alcohol (70, 80, 95, and 100% ethanol in twice distilled water), and then for 60 min in each of two 100% ethanol washes. Cultures were transferred to vials containing 100% acetone. After three changes of acetone, each over 1 h, cultures were infiltrated overnight with a 1:1 mixture of acetone and epon-araldite. The next day the acetone was allowed to evaporate off in a fume hood, cultures were placed in fresh epon-araldite for ~6 h, and finally transferred to a mould in a 70°C oven to polymerize overnight. All procedures were carried out at room temperature.

For light microscopic examination, sections were cut 1 μm thick with a dry glass knife using an LKB ultratome. The sections were mounted on glass slides and stained with 1% toluidine blue in 1% borax. Sections were examined with a Leitz Orthoplan microscope. When endothelial cells containing lumens were seen the block was trimmed for thin sectioning (EM). For EM, thin sections of silver interference color were cut with a Swiss diamond knife (Diatome Ltd., Fort Washington, PA), and picked up on clean uncoated 200-mesh copper grids. Sections were stained for 5 min with 2% uranyl acetate in 50% ethanol, and for 5 min with lead citrate. Micrographs were taken on a Philips 410 electron microscope at 80 Kv. Magnifications were determined by means of a carbon grating replica. At both the light microscope level and the electron microscope level, serial sections up to 30 each (1 μm thick or silver interference color) were taken to establish that the lumens were continuous through the endothelial cell.

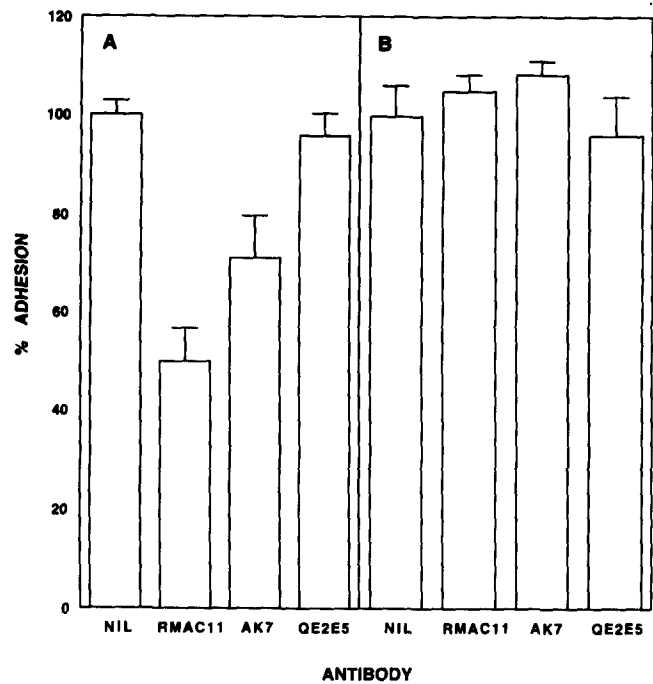


Figure 1. Anti- $\alpha_2\beta_1$ antibodies partially inhibit EC adhesion to collagen I and not to fibronectin. 5×10^3 EC in 50 μl of serum-free medium were added to microtiter wells which had previously been coated with either collagen I (A) or fibronectin (B). The antibodies, as indicated, were added at a final concentration of 30 $\mu\text{g/ml}$. The plates were incubated at 37°C for 2 h, washed, and the number of attached cells assessed by the methylene blue assay (as outlined under Proliferation Assay in Materials and Methods). The results were normalized where 100% adhesion is taken as the OD630 nm in wells without antibody. The results show the mean of triplicate wells for each group in one experiment representative of five similar experiments. $p < 0.001$ for groups with RMAC11 and AK7 compared to no antibody group on the collagen I matrix.

Statistics

Significance was determined by the ANOVA test for analysis of variance or by the unpaired t test.

Results

Inhibition of Endothelial Cell Adhesion by Anti-Integrin Antibodies

Adhesion of HUVEC to a collagen I matrix was inhibited, although only partially, by RMAC11 and AK7 (Fig. 1 A) but the antibodies had no effect on HUVEC attachment to fibronectin (Fig. 1 B) or gelatin (data not shown). Cells did not attach to laminin in the absence of FCS. The antibody QE2E5 which binds to the β_1 chain had no effect on the attachment of cells to any of the matrices tested. However, two other β_1 specific antibodies, P4C10 and 612C4, almost totally inhibited HUVEC adhesion to collagen (and fibronectin) (data not shown).

HUVEC Plated onto Collagen Gels Are Induced to Form Capillary Tubes

EC, when plated onto a gel of collagen are induced to form a capillary network within the collagen gel in the presence of PMA (Fig. 2, B and C) (Montesano et al., 1983; Mon-

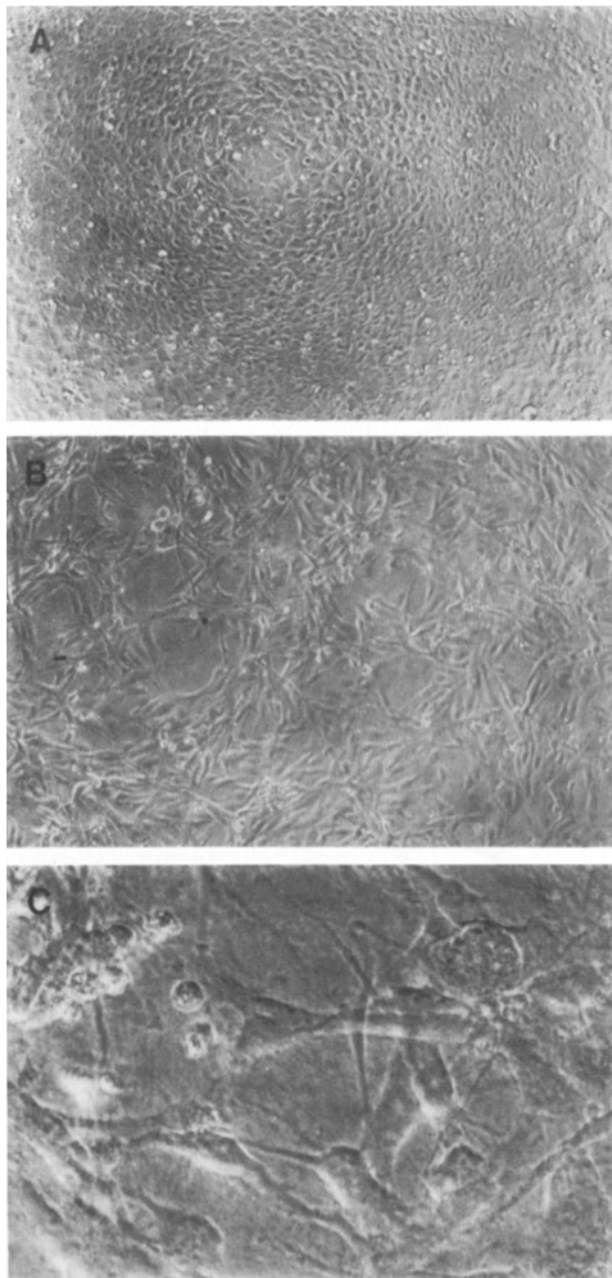


Figure 2. EC plated on collagen I gel in the presence of PMA are induced to form capillary tubes. 6.4×10^4 EC/well in $160 \mu\text{l}$ of HUVEC medium either in the absence (A) or presence (B) of 20 ng/ml PMA were plated onto a gel of collagen I formed in microtiter wells. The results were visualized 24 h after cell plating. C is a high power photograph of group B. Photographs show representative fields of one well of duplicate wells set up for each group. Magnification (A) $\times 60$, (B) $\times 60$, and (C) $\times 240$.

tesano and Orci, 1985). Realignment of the EC, cell invasion into the gel, and the beginning of cell elongation are evident ~ 8 h after plating of the cells onto the gel, and tube formation was clearly visible by 12 h. Very little, if any, tube formation occurs in collagen gels in the absence of PMA or with addition of dimethylsulphonic acid, used as a carrier for PMA; the cells are maintained as a monolayer on top of the gel and little invasion into the gel is seen (Fig. 2 A). Tube formation was highly dependent on the concentration of cells

plated onto the collagen gel, no tubes being observed with less than $1-2 \times 10^4$ cells/well suggesting that cell-cell contact is important.

Analysis by EM of EC, 24 h after plating on collagen gels with the addition of PMA showed the presence of vacuole-like structures similar to those described by Folkman and Haudenschild (1980) (Fig. 3 A). A continuous membrane surrounded each vacuole which was either empty or filled with amorphous material. Fusion of these vacuoles with the plasma membrane was occasionally observed. Serial sectioning to $>30 \mu\text{m}$ and visualization at the light microscopy level showed a continuous vacuole-like structure confirming that these were indeed lumina. At least 70% of all cells visualized showed vacuolization. Only the occasional lumen was formed from multiple cells.

Effect of Anti-Collagen Receptor Antibodies on Capillary Formation

EC were plated onto collagen gels in the presence of PMA with increasing concentrations of antibodies RMAC11, AK7, and QE2.E5. Capillary formation was assessed 24 h later. Fig. 4 A shows the capillary tube formation taking place with PMA alone. No change in the extent of tube formation was seen with the addition of QE2E5 (Fig. 4 B). However, a more extensive capillary network was seen in wells containing RMAC11. The tubes appeared longer and wider, and less monolayer was evident (Fig. 4 C). Similar results were obtained with AK7, P4C10, and 612C4 and with EC derived from neonatal foreskins (data not shown). The enhancement of tube formation with RMAC11 over PMA alone was clearly evident in the high power photographs (Fig. 5, A-F). Tube number, length, and width were quantified from high power photographs and results from four separate experiments were analyzed and pooled and are shown in Fig. 6. The anti- $\alpha_2\beta_1$ antibody RMAC11 clearly enhanced the length (A), width (B), and number (C) of tubes.

EM showed that in the presence of RMAC11, cells were seen in larger aggregates suggesting cell-cell adhesion was promoted (not shown). Furthermore, larger lumina were evident compared to PMA group only, the majority of which appeared to be formed from multiple cells as shown in Fig. 3 B. Intercellular junctions are clearly visible at the EM level and by analysis of these junctions the lumen shown in Fig. 3 B is formed from five EC. Analysis of thin sections (silver interface color) taken through gels showed that with PMA $73 \pm 5.7\%$ (mean \pm SEM) of lumina were formed from single cells (i.e., intracellular lumen, Fig. 3 A). The remaining lumina were formed from two or three cells. In contrast, in the presence of RMAC11, $83 \pm 7.4\%$ of lumina were formed from three or more cells (values were obtained from four separate experiments with 50 lumen examined in each group in each experiment). Thus the increase in capillary thickness appears to be due to the conversion from intracellular to multicellular lumina.

Maximum levels of tube formation were seen with 30 $\mu\text{g/ml}$ RMAC11 (Table I) but some enhancement over the level seen with PMA alone was normally observed with 3 $\mu\text{g/ml}$ RMAC11. Tube length, width, and number were assessed visually through different focal plains of the gel and assigned values from + to ++++. Tube length, width, and number were also measured from high power photographs.

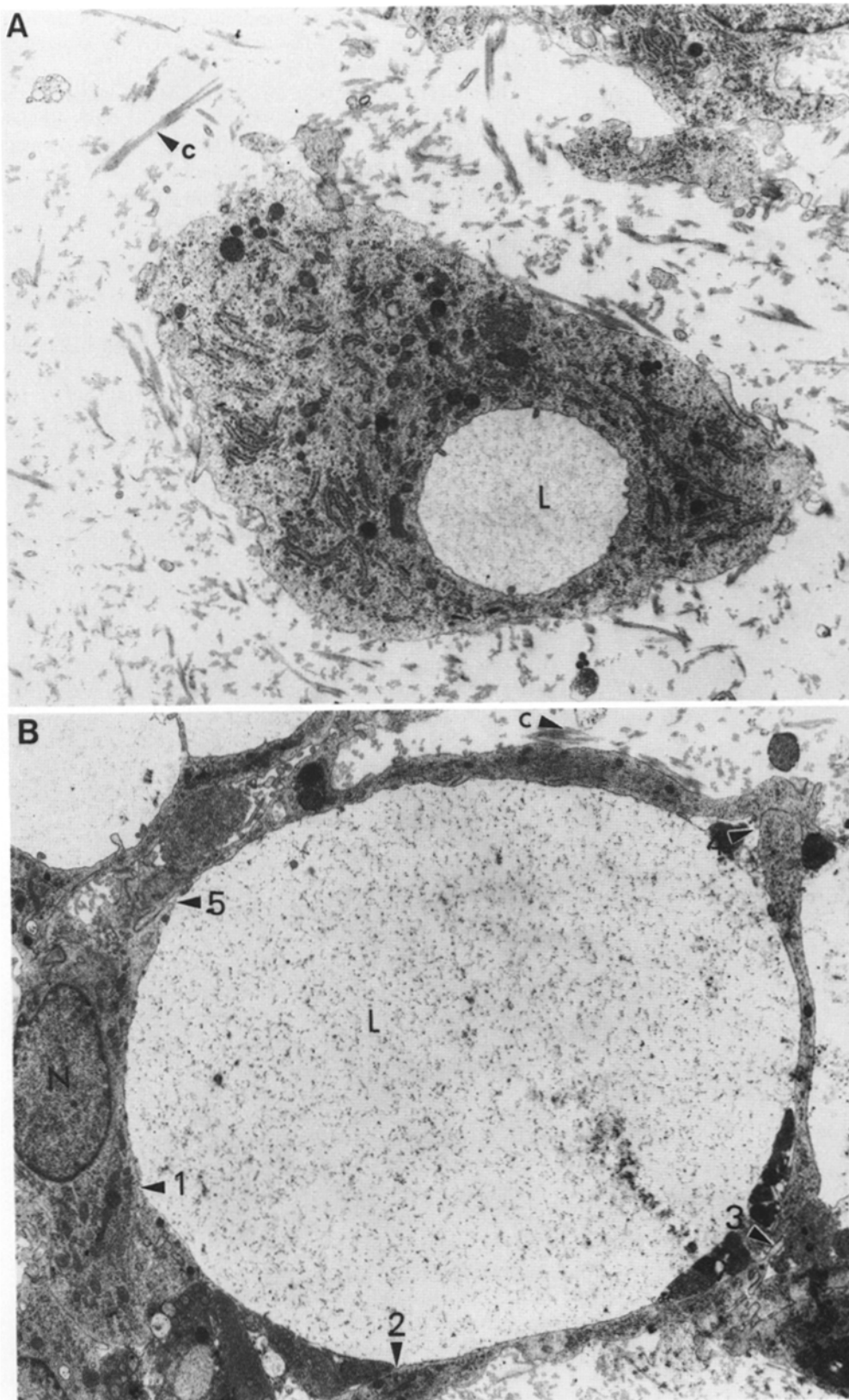


Figure 3. Formation of lumen-containing capillaries in collagen gels. EC in the presence of 20 ng/ml PMA (A) or PMA and 30 μ g/ml RMAC11 (B) were plated onto a collagen gel. 24 h later the cells were fixed, embedded in epon-araldite, and sectioned for EM. Magnification (A) \times 7490 and (B) \times 4000. C, collagen; L, lumen. Numbers 1-5 relate to cell junctions.

As is seen in Table I, the two assessments for capillary formation showed a good correlation. Analysis of EC by flow cytometry showed that 30 μ g/ml of RMAC11 fully saturated the $\alpha_2\beta_1$ receptor binding sites (data not shown). With

AK7, an enhancement of tube formation was also seen over the concentration range of 3-30 μ g/ml. However, the extent of enhancement was never as great as that seen with RMAC11.

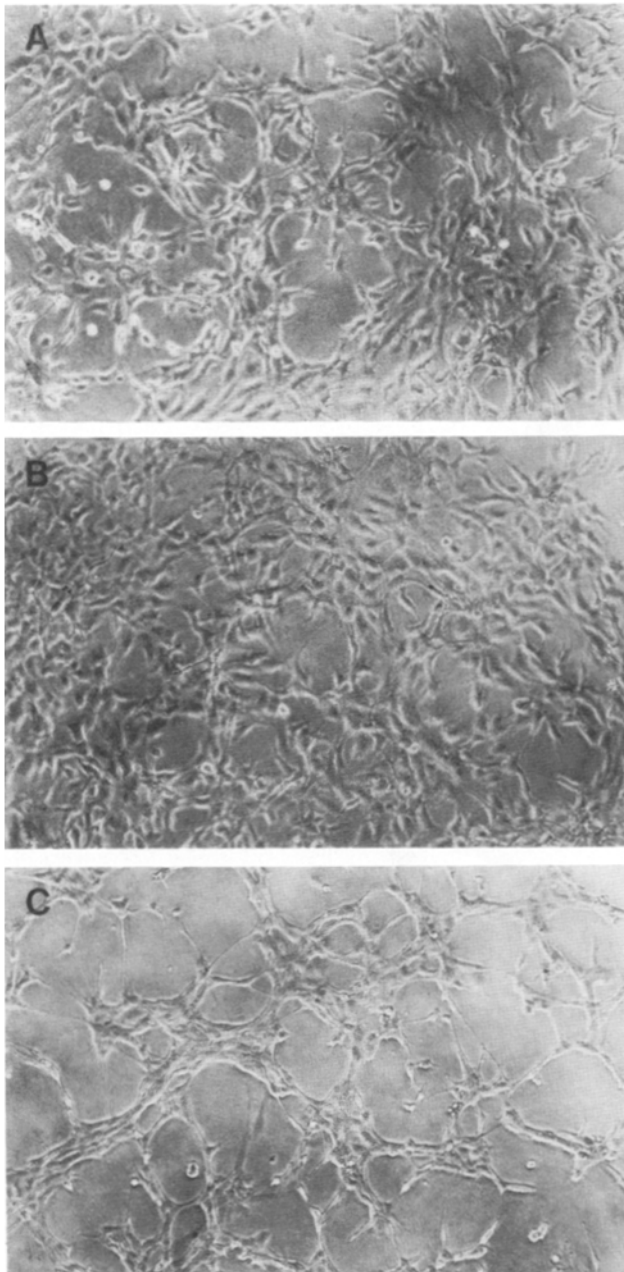


Figure 4. Anti- $\alpha_2\beta_1$ antibodies enhance capillary tube formation in collagen gels. EC were plated onto collagen gel in the presence of 20 ng/ml PMA. To group B, QE2E5 was added at plating and to group C, RMAC11 both at a final concentration of 30 μ g/ml. Groups were assessed for tube formation 24 h later. Each photograph shows a representative field taken from one well of duplicate wells set up for each group. Similar results have been obtained more than 20 times using different EC lines. Magnification $\times 60$.

The effect observed by RMAC11 and AK7 is unlikely to be due to nonspecific effects via Fc receptor mediated events since Fab₂ fragments of RMAC11 exhibit a similar enhancement of tube formation as the whole Ig (data not shown). Furthermore, since Fab' fragments of RMAC11 were also able to enhance tube formation (data not shown), cross-linking of the antigen is also not likely to be responsible for the enhancement.

Pretreatment of the HUVEC with RMAC11 or AK7 for 15

min either at room temperature or 37°C followed by washing to remove unbound antibody, did not result in enhanced tube formation (data not shown).

The enhancement of tube formation was dependent on the addition of RMAC11 or AK7 within the first 2 h of cell plating. If the antibodies were added after this, no enhancement of tube formation was evident (data not shown).

Angiogenesis can also be induced in collagen gels in the presence of PMA by resuspending the cells within the gels rather than layering them on top of the gel (Madri et al., 1988). Pretreatment of EC with PMA and RMAC11 before resuspension within the gel led to enhancement of tube formation over that seen with PMA alone (data not shown). Thus, the anti- $\alpha_2\beta_1$ antibodies were able to enhance tube formation whether the EC contact collagen in a polarized fashion or whether the cells are totally surrounded by the collagen matrix.

Anti- $\alpha_2\beta_1$ Antibodies Induce Capillary Tube Formation in Rigid Gels

The ability of RMAC11 and AK7 to enhance capillary tube formation was most strikingly evident when EC were plated onto rigid collagen gels formed after the collagen was gelled for 1 h at 37°C. Even in the presence of PMA, very little, if any, tube formation is seen with these rigid gels and the cells maintain a flat cobblestone appearance on top of the gel. However, the addition of RMAC11 (or AK7) with PMA overcomes the inhibitory effect caused by the rigidity of the gel; the cells invade into the gel and capillary tubes and an anastomosing network are visible (Fig. 7, A–D) although not to the same extent as is normally seen on less rigid gels. Measurements of tube numbers from random high power fields from five separate experiments on rigid gels each using a different HUVEC line were made. The mean tube number with PMA alone was 1.0 ± 0.4 (mean \pm SEM) and with RMAC11 was 39.2 ± 4.08 ($p < 0.0005$) clearly demonstrating the promotional activity of RMAC11. One possibility for the antibodies enhancing the ability of the EC to breakdown and invade the gel is by an increase in the synthesis of matrix-degrading enzymes such as collagenase. However, no change in the total level of active collagenase was observed either in normal gels or in rigid gels. The level of detection of collagenase by the assay used (see Materials and Methods) was 10 ng/ml. In a representative experiment, the level of collagenase induced on collagen gels in the presence of 20 ng/ml PMA was 112 ± 5.9 and with PMA and RMAC11 120 ± 2.0 (mean \pm SEM, $n = 3$).

Effect of Anti- $\alpha_2\beta_1$ Antibodies on EC Morphology

As was shown in Fig. 1, the addition of RMAC11 and AK7 to HUVEC cultured on 2-D substrates of collagen, but not on gelatin or fibronectin, resulted in a decrease in the number of cells attached when measured 2 h after plating. No differences were seen in the number of cells attached when measured at 4, 6, or 24 h after plating. On collagen gels, in the presence of QE2E5, the cells had become flattened and were beginning to adopt their characteristic cobblestone morphology (Fig. 8 A) when viewed 2 h after plating. In the presence of RMAC11 (or AK7), cells remained rounded (Fig. 8 B) for ~ 2 –3 h after which time they flattened and no differences from control wells were seen.

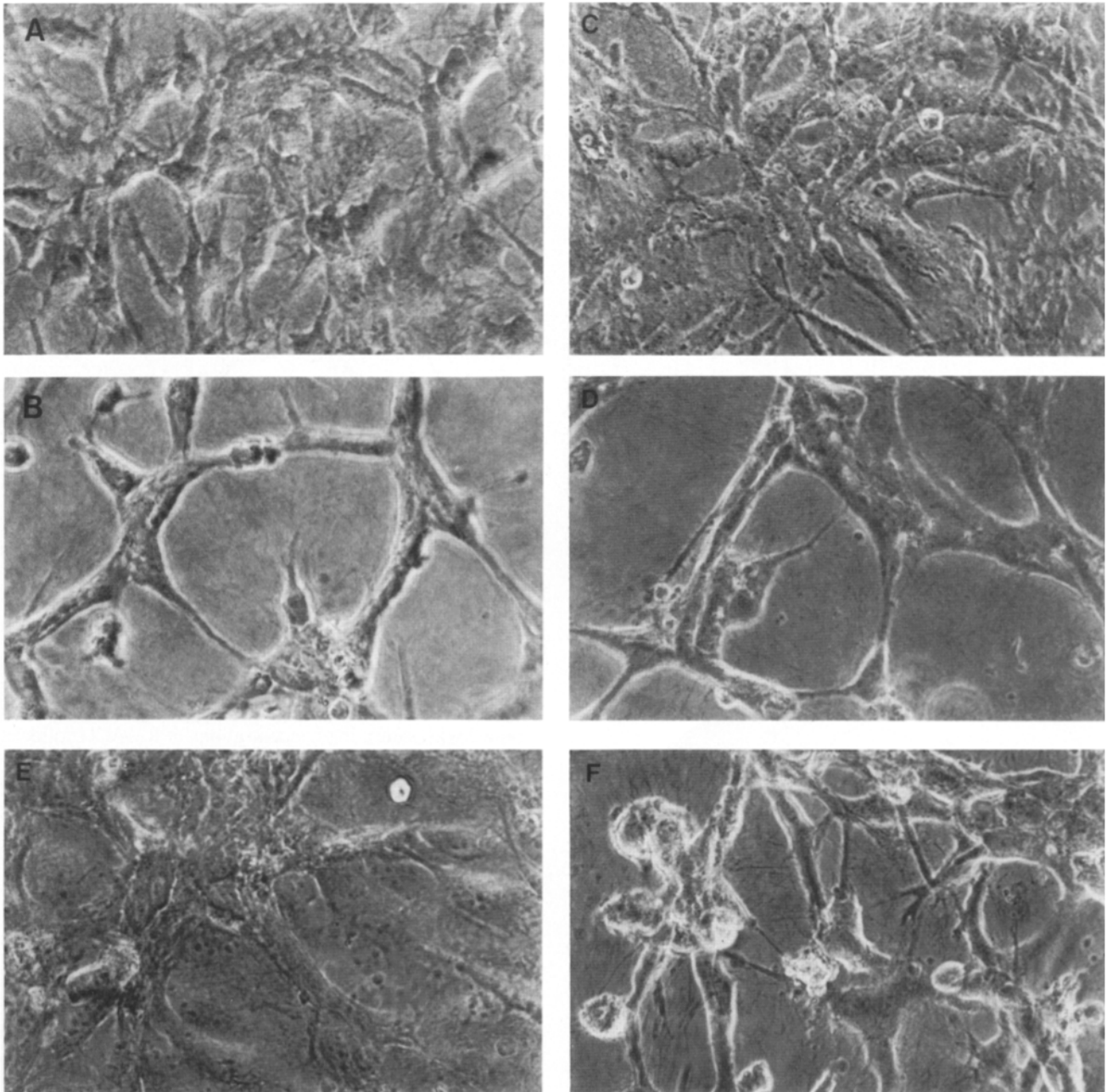


Figure 5. High power photographs of capillary tubes formed on collagen gels in the presence of anti-integrin antibodies. EC were plated onto collagen gel in microtiter wells with 20 ng/ml PMA. *A* and *B* show one EC line, *C* and *D* another line, and *E* and *F* a third line. To one group (*A*, *C*, and *E*), 30 $\mu\text{g}/\text{ml}$ of QE2E5 was added and to the other group (*B*, *D*, and *F*), 30 $\mu\text{g}/\text{ml}$ RMAC11. All wells were incubated for 24 h. Each photograph shows a representative field taken from one well of duplicate wells set up for each group. Magnification $\times 264$.

Anti- $\alpha_2\beta_1$ Antibodies Inhibit Endothelial Cell Proliferation

Alterations in cell shape can have profound effects on proliferation, and EC proliferation is essential for tube extension although not required for initial sprouting (Sholley et al., 1984; Folkman, 1982). The proliferative response of HUVEC in the presence of RMAC11 and AK7 was therefore measured. A decrease in EC proliferation in the presence of RMAC11 or AK7 was seen when the cells were plated on a 2-D matrix of collagen. This was clearly seen with RMAC11

when the assay was performed in the presence of either 20 (data not shown) or 2% FCS (Fig. 9). AK7, induced a significant level of inhibition only when 2% FCS was used in the assays and this level of inhibition was less than that induced by RMAC11. The results in Fig. 9 have been normalized to the no antibody control. When the actual cell numbers were counted in wells containing 30 $\mu\text{g}/\text{ml}$ QE2E5, AK7, or RMAC11 the increase in cell numbers was 2.6, 2.0, and 1.4-fold, respectively. These results clearly show that the ability of antibodies to inhibit proliferation appears to correlate with enhancement of capillary formation. No inhibition

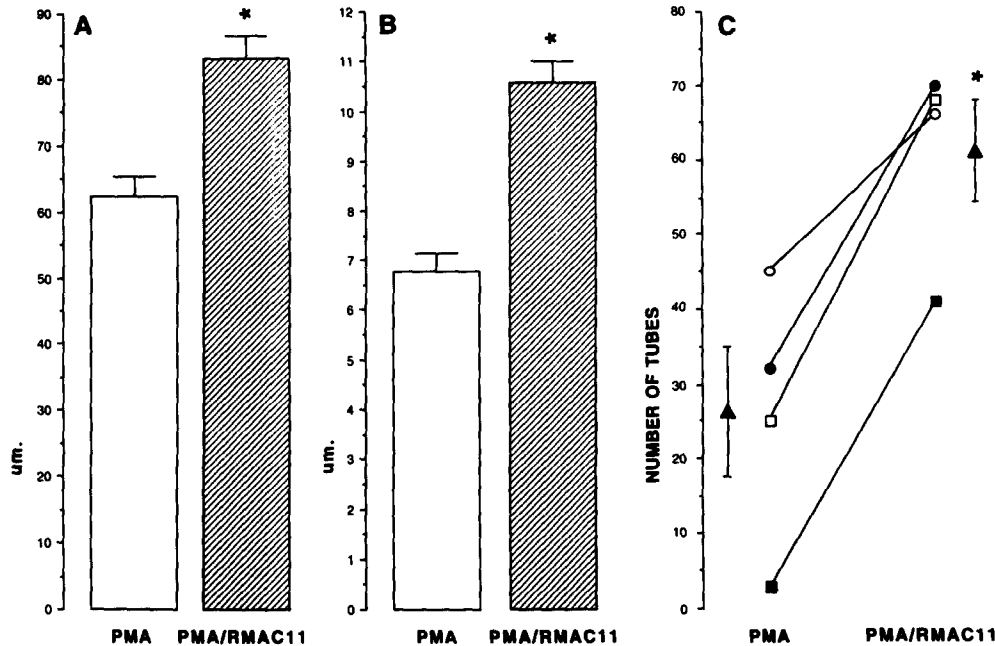


Figure 6. Effect of RMAC11 on tube length (μm A), width (μm B), and number (C) per high power field. EC were plated onto collagen gels in the presence of 20 ng/ml PMA. 30 $\mu\text{g}/\text{ml}$ of RMAC11 was added to half the wells. Each group contained duplicate wells. 24 h later the wells were photographed (magnification $\times 264$), random fields being taken for each well. From these photographs, the number, length, and width of tubes were calculated. The pooled results from four separate experiments using four different EC lines are shown for tube length and width (mean \pm SEM). Each experiment showed a similar and significant increase with RMAC11. In C, the tube numbers for each experiment are shown together with the mean \pm SEM (Δ) for the four experiments. * $p < 0.005$ compared to PMA alone.

of proliferation was seen with either antibody when the cells were plated onto fibronectin or gelatin (data not shown).

PMA Does Not Alter Expression of $\alpha_2\beta_1$ on Endothelial Cells

One possibility for the effect of anti- $\alpha_2\beta_1$ antibodies on capillary tube formation in collagen gels is that PMA may alter the surface expression of the $\alpha_2\beta_1$ molecule. To investigate this, HUVEC were plated onto collagen-coated microtiter wells at numbers to give either a confluent monolayer (that is nonproliferating) or a semiconfluent monolayer (that is to give a proliferating population) either in the presence or absence of 20 ng/ml PMA. HUVEC were also plated onto collagen gels in the presence or absence of PMA. 24 h later, the

cells were detached from the microtiter wells with trypsin or extracted from the gels with collagenase. The cells were stained with saturating concentrations of RMAC11 followed by a fluorescein-conjugated sheep anti-mouse Fab₂ antibody. There was no difference in the mean channel fluorescence between confluent and semiconfluent cells either in the presence or absence of PMA, or between tube forming or nonforming cells suggesting that PMA within this time period did not alter the level of surface expression of $\alpha_2\beta_1$ on HUVEC.

Anti-VnR Antibodies Enhance Capillary Formation in Fibrin Gels

Capillary tube formation also takes place in fibrin gels in the presence of PMA (Montesano et al., 1987). Adhesion of EC to fibrinogen is mediated through another integrin complex, the $\alpha_v\beta_3$ (or vitronectin receptor, VnR). To determine whether tube formation in fibrin gels is enhanced in the presence of antibodies which limit cell-matrix interactions, we used two antibodies (LM609 or 13C2) which are directed to the $\alpha_v\beta_3$ complex and known to inhibit EC-fibrinogen adhesion (Cheresh and Spiro, 1987 and unpublished data). Fig. 10 shows that $\alpha_v\beta_3$ is also involved in the adhesion of HUVEC to fibrin, since anti- $\alpha_v\beta_3$ antibody (LM609) partially inhibits attachment of HUVEC to fibrin-coated plastic (A) but not to collagen (B). Similar results were obtained with 13C2 (data not shown). As seen in Fig. 11, tube formation is enhanced in the presence of LM609 and similarly for 13C2 (data not shown) although the level of tube formation was never as large as that seen in collagen with either RMAC11 or AK7. Functional effects with LM609 were observed with concentrations as low as 3 $\mu\text{g}/\text{ml}$. In the initial 1-2 h on fibrin gels, cells incubated with anti- $\alpha_v\beta_3$ antibody remained rounded and failed to flatten compared to cells

Table I. Antibody RMAC11 Enhances Capillary Tube Formation in a Dose-dependent Manner

Concentration of RMAC11 ($\mu\text{g}/\text{ml}$)	Measurement of capillary tubes			Capillary tube formation (+ to +++)
	Number/field	Length (μm)	Width (μm)	
0	7	43.9 \pm 2.4	6.1 \pm 0.5	+
0.3	13	46.5 \pm 3.7	7.6 \pm 0.9	++
3	33	52.6 \pm 5.4	11.0 \pm 1.6	+++
10	50	81.8 \pm 8.0	12.5 \pm 0.8	+++
20	87	70.0 \pm 4.5	11.7 \pm 0.9	++++
30	91	75.4 \pm 7.5	11.0 \pm 0.7	++++

EC were plated onto a collagen gel in the presence of 20 ng/ml PMA together with varying concentrations of RMAC11. Measurement of the number, length, and width of tubes was made from high power photographs taken at random from each well, the measurements for length and width are given as mean \pm SEM. The extent of capillary tube formation was also assessed and is given as + to ++++ based on the length, width, and number of capillary tubes. The results are shown for one experiment which was similar to three performed, each with a different EC line.

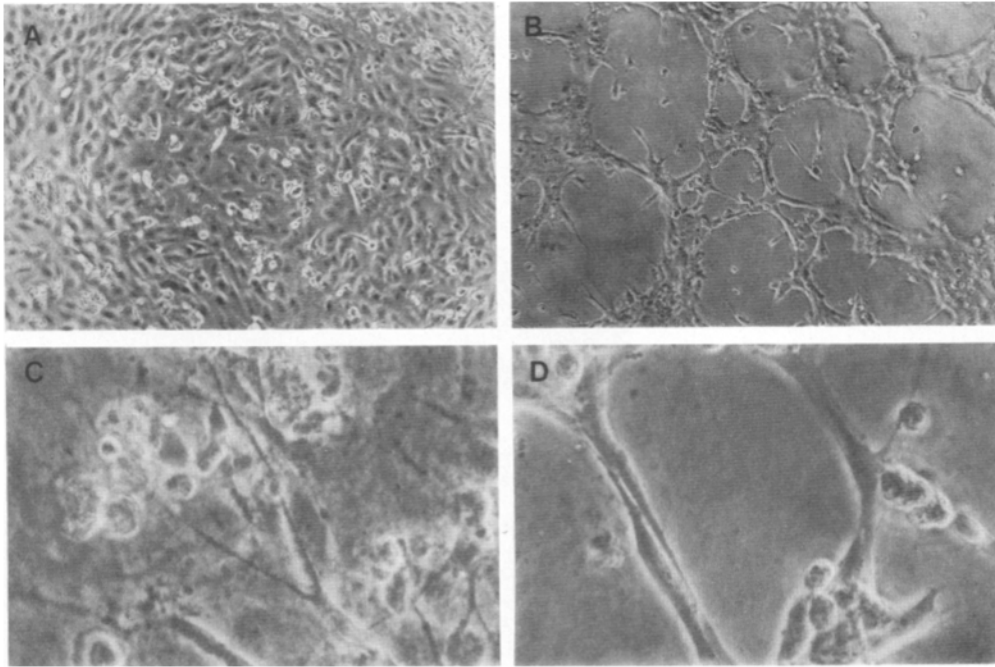


Figure 7. Anti-integrin antibodies promote tube formation in rigid collagen gels. EC were plated onto collagen which had been allowed to gel for 1 h at 37°C giving a more rigid gel than that obtained after our normal gelling time of 10–20 min. *C* and *D* are high powered views of *A* and *B*, respectively. 20 ng/ml of PMA was added to both groups, 30 μ g/ml of RMAC11 was added to *B*. Tube formation was assessed after 24 h. *A* and *B* magnification $\times 80$; *C* and *D* magnification $\times 310$. Each is a representative field of one well of duplicate wells set up for each group. The experiment has been performed at least six times using different EC lines and using either RMAC11 or AK7 with similar results being obtained.

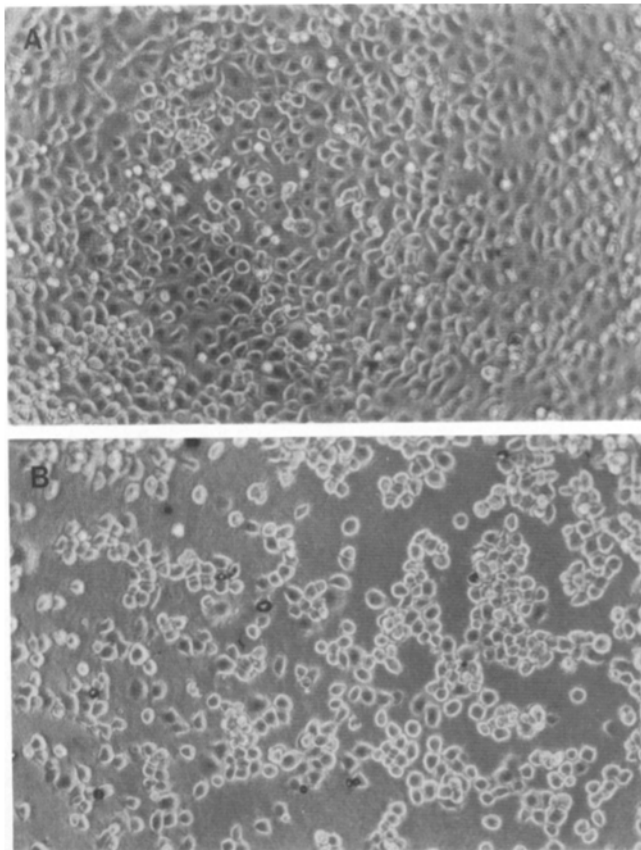


Figure 8. Anti-integrin antibodies maintain EC in a rounded morphology and prevent cell spreading. EC were plated onto collagen I gels with 20 ng/ml PMA either in the presence of 30 μ g/ml QE2E5 (*A*) or 30 μ g/ml RMAC11 (*B*). The cells were viewed at 1, 2, 3, 4, and 6 h after plating. These photographs were taken at 2 h after plating (magnification $\times 110$). Each shows a representative field of one well from duplicate wells set up for each group. The experiment has been performed on at least four separate EC lines with similar results.

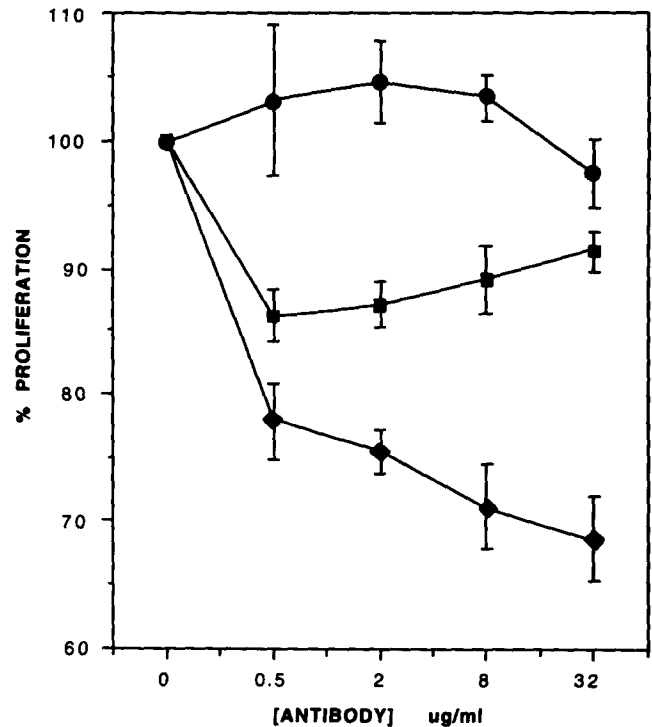


Figure 9. Anti- $\alpha_2\beta_1$ antibodies inhibit EC proliferation. EC were plated onto collagen I-coated microtitre wells at 5×10^3 cells/well in HUVEC medium containing 2% FCS either with QE2E5 (\bullet), RMAC11 (\blacklozenge), or AK7 (\blacksquare) at various concentrations. The cells were incubated for 3 d at 37°C, washed, fixed, stained with methylene blue, and the dye solubilized with ethanol. Absorbance was read at 630 nm. The OD₆₃₀ of wells with no antibody was taken to give 100% proliferation. All other groups were normalized to this. The results show the mean \pm SEM of four experiments where each point in each experiment was performed with six replicates. Groups containing RMAC11 and AK7 were significantly different ($p < 0.0001$) from groups containing QE2E5 (ANOVA test for significance).

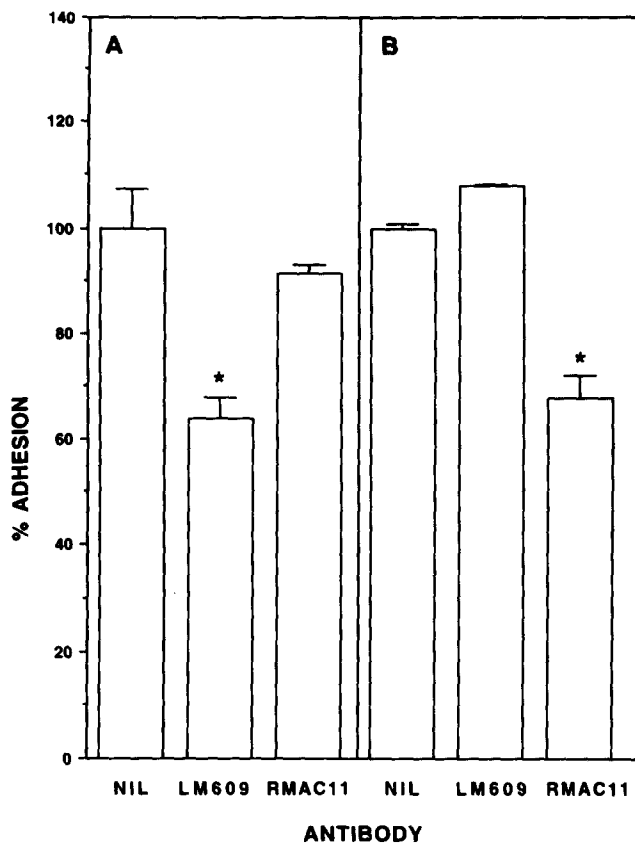


Figure 10. Anti- $\alpha_v\beta_3$ antibody partially inhibits EC adhesion to fibrin (A) but not to collagen (B). Fibrin-coated microtiter wells were formed by thrombin cleavage of fibrinogen followed by two washes in medium containing FCS. Antibodies as indicated were added at a final concentration of 30 $\mu\text{g/ml}$ to either fibrin (A) or collagen (B)-coated wells. The plates were incubated at 37°C for 1 h, washed, and the number of attached cells assayed as given for Fig. 1. The results show the mean of triplicate wells for each group of one experiment representative of three experiments. $p < 0.01$ compared to no antibody group on either matrix.

without antibody. Sections analyzed by light microscopy showed details similar to that observed in collagen gels. With PMA alone, small lumen formed by intracellular vacuolization were seen. In the presence of LM609 or 13C2 there was extensive cell-cell interactions and lumina were formed between cells. Fig. 12 shows that in the presence of anti- $\alpha_v\beta_3$ antibody, the length, width, and the number of tubes were increased over that seen with PMA alone. Analysis of thin sections showed that with PMA alone, the majority of lumen were formed from single intracellular lumen although lumina formed from two cells were occasionally seen. In the presence of anti- $\alpha_v\beta_3$ antibody, the majority of lumina (>70%) were formed from three cells with some lumina formed from four or five cells. The enhancement of tube formation seen with $\alpha_2\beta_1$ antibodies and with anti- $\alpha_v\beta_3$ antibodies was specific for the matrix used; $\alpha_2\beta_1$ antibodies had no effect in fibrin gels and $\alpha_v\beta_3$ antibody had no effect in collagen gels (data not shown).

Discussion

The central finding of this study is that anti-integrin antibod-

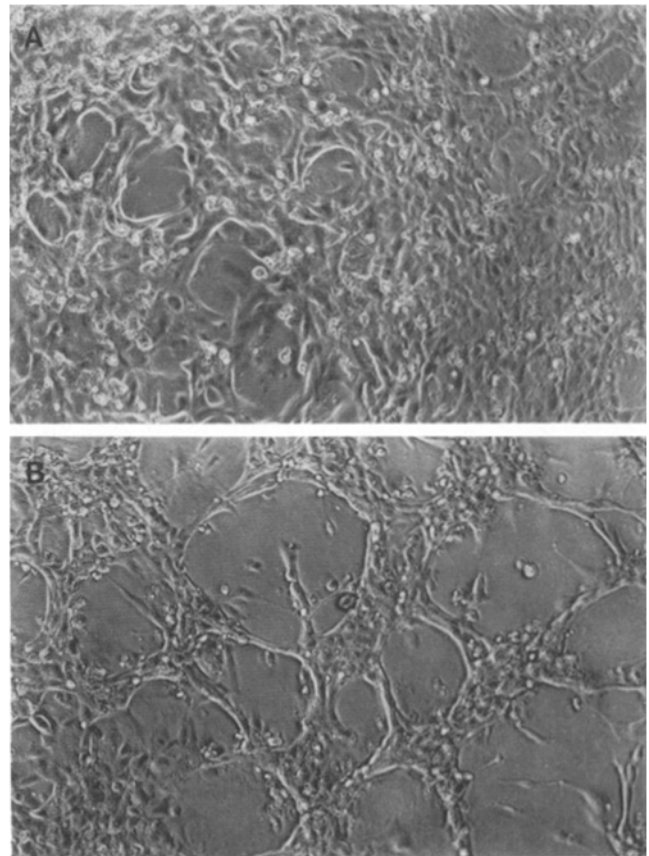


Figure 11. Anti- $\alpha_v\beta_3$ antibody promotes capillary formation in fibrin gels. 6.4×10^4 EC/well were plated onto fibrin gels in the presence of 20 ng/ml PMA. The anti- $\alpha_v\beta_3$ antibody LM609 at a final concentration of 30 $\mu\text{g/ml}$ was added to group B. Cells were incubated overnight. A representative field of one well of duplicate wells in each group is shown (magnification $\times 110$). The experiment has been performed at least five times using a different EC line for each experiment, all giving similar results.

ies are able to enhance the formation of capillary tubes in vitro. Functional monoclonal antibodies directed to the major integrin receptors for the tube-permissive matrices of collagen and fibrin enhanced capillary tube formation increasing the number, length, as well as width. Ingber and Folkman (Ingber and Folkman, 1989a,b; Ingber, 1990, 1991a,b) propose that changes in adhesivity (e.g., by altering the density of ECM molecules), and therefore in the ability of the matrix to resist cell tension, may result in alteration in cell function such as proliferation and differentiation. Thus the mechanical forces between cells and their environment will govern their behavior. Our results showing that anti-integrin antibodies, which block adhesion, enhance tube formation suggest that angiogenesis may also be regulated by the adhesivity of EC for the matrix. The balance between cell and matrix adhesivity will clearly be important in determining the function of endothelial cells.

In the presence of antibody (anti- $\alpha_2\beta_1$ and anti- $\alpha_v\beta_3$), the first change observed was that the EC remained rounded, became less adhesive, and failed to spread when plated onto gels or 2-D matrices rather than adopting their normal cobblestone, flattened morphology. It is known that alteration in cell shape can have profound effects on the function of many

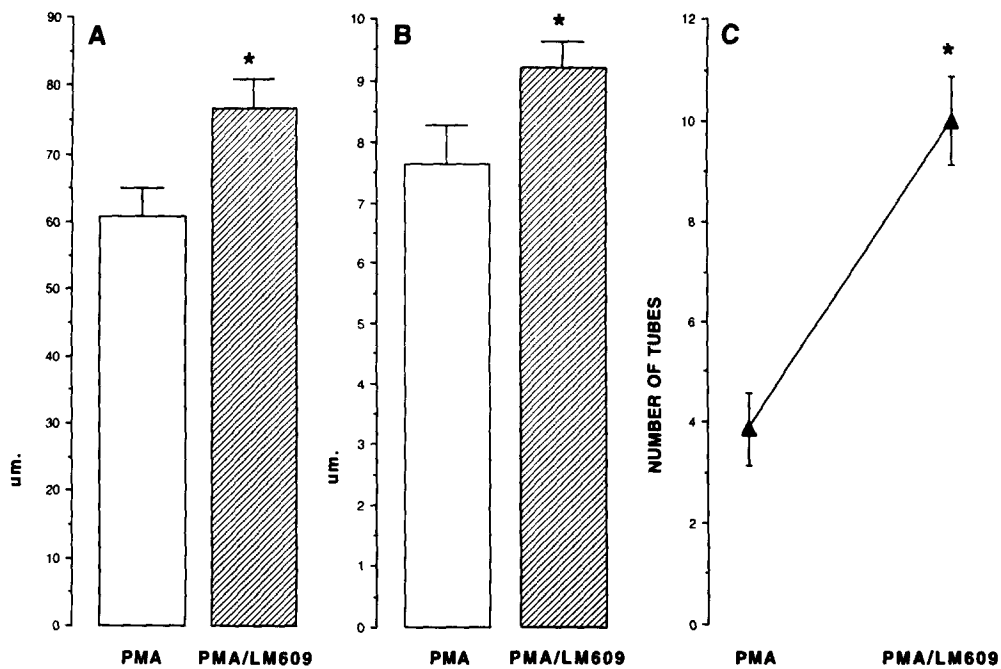


Figure 12. Effect of LM609 on tube length (μm , A), width (μm , B), and number (C) per high power field. EC were plated onto fibrin gels in the presence of 20 ng/ml PMA, and 30 $\mu\text{g}/\text{ml}$ LM609 was added to half the wells. 24 h later the wells were photographed (magnification $\times 264$), random fields being taken for each well. The number, length, and width of tubes were measured from these photographs. The pooled results from two separate experiments are given for the length, width, and number. * $p < 0.05$ compared to PMA alone.

cells resulting in changes to cell proliferation and differentiation (Folkman and Moscona, 1978). Gospodarowitz has reported that EC must become attached and flattened in order to proliferate and that those kept rounded in suspension fail to divide (Gospodarowitz et al., 1978). Furthermore, there is a direct correlation between growth inhibition and decreases in cell extension (Ingber et al., 1987). DNA, RNA, and protein synthesis in anchorage dependent fibroblasts is also inhibited if these cells are maintained in suspension (Ben-Ze'ev, 1980). Differentiation and expression of genes which reflect a more differentiated state of hepatocytes, chondrocytes, fibroblasts, and endothelial cells are linked to cell shape and actin reorganization (Aggeler et al., 1984; DiPersio et al., 1991; Mallein-Gerin et al., 1991; Ingber and Folkman, 1989a,b; Ben-Ze'ev et al., 1988; Glowacki et al., 1983; Unemori and Werb, 1986; Werb et al., 1986). All these studies suggest that the mechanical interaction of cells with the ECM can regulate cell function. Since integrins interconnect the ECM with the cyto-skeleton, they are likely to be involved in the transmission of signals between the cell and its ECM. The biochemical signals (termed mechanotransducers) which are generated as a result of mechanical forces or integrin activation are unknown at present but phosphorylation (Kornberg et al., 1991), activation of the Na^+/H^+ exchanger (Ingber, 1990), Ca^{++} mobilization, and adenylate cyclase (for review see Watson, 1991; Ingber, 1991b) have been implicated.

In the studies reported here, the decrease in the adhesion and change in cell shape of EC with anti-integrin antibodies, resulted in an inhibition in proliferation and in a promotion of differentiation as measured by capillary tube formation. These alterations in cell function were matrix specific, that is anti- $\alpha_2\beta_1$ antibodies inhibited EC proliferation and enhanced cell differentiation only on a collagen but not on a fibrin gel, while anti- $\alpha_v\beta_3$ showed effects on fibrin but not on collagen gels. Thus, the effect of anti-integrin antibodies on angiogenesis is ligand dependent. Furthermore, the antibody mediated effects are time dependent. No enhancement

of capillary formation was observed when the antibodies were added more than 2 h after cell plating, paralleling the time dependency seen with the antibodies on EC adhesion and cell shape.

EM revealed striking qualitative changes in tubes with anti-integrin antibodies. In the absence of RMAC11, the majority of lumina were formed within single cells in a manner reported by Folkman and Haudenschild (1980). However, with RMAC11, the majority of lumina were formed from multiple cells with clear cellular borders between cells making up the vessel (Fig. 3 B). Using fibrin gels and anti- $\alpha_v\beta_3$ antibodies, lumina were also formed from multiple cells. Thus anti-integrin antibodies not only alter the degree of tube formation taking place but also influence the phenotype of tubes. The relationship between intracellular and intercellular lumina is not known at present but one possibility is that intercellular lumen form from coalescence of intracellular vacuole-like structures with the plasma membrane and that these structures define stages in tube formation. Clearly, our in vitro model of angiogenesis may allow a more detailed examination of the stages involved in capillary formation.

The enhancement of capillary tube formation with anti-integrin antibodies was dependent on the presence of PMA since the antibodies alone had no effect. Thus, one signal for tube formation is likely to be protein kinase C-dependent. Indeed activators of protein kinase C inhibit the proliferation of EC in response to mitogens (Doctrow and Folkman, 1987). One possibility for our results is that the antibodies enhance PMA-mediated signals. This, however, is unlikely since the antibodies do not enhance tube formation on an inappropriate matrix. That is, anti- $\alpha_2\beta_1$ antibody had no effect on fibrin gels, and anti- $\alpha_v\beta_3$ antibody had no effect on collagen gels even though tube formation can take place on these matrices. This data therefore suggests that the antibodies do not directly signal the cell to undergo tube formation. This is further supported by the fact that pretreatment of HUVEC with the antibodies and removal by washing does not result in enhanced tube formation.

No capillary tube formation is induced on collagen gels that are too rigid even in the presence of PMA. The cells form a confluent monolayer and little or no invasion into the matrix takes place suggesting that EC differentiation was inhibited by the rigid ECM. However, anti- $\alpha_2\beta_1$ antibody on rigid gels in the presence of PMA did induce EC invasion into the gel and subsequent tube formation. The cells in the presence of RMAC11 were more rounded than with control antibody and failed to spread and flatten. A consequence of changes in cell shape, adhesion, and signaling may be an alteration in the level of matrix degrading enzymes such as collagenase. However, we observed no change in the total collagenase produced by the EC in the presence of RMAC11. One possibility to explain these results is that there is an alteration in the site of collagenase release rather than an alteration in the overall level of production. Enzyme redistribution has been demonstrated for urokinase plasminogen activator (uPA) after anti-fibronectin antibody binding to rabbit fibroblasts (Werb et al., 1989). Thus, as a consequence of antibody-integrin binding (and perhaps integrin redistribution and change in cell shape) collagenase may be redirected to specific localized areas resulting in enhanced cell motility and gel invasion. In addition, since activation of collagenase can occur via cleavage by uPA measurement of uPA or its receptor may indicate altered enzyme activity (Mignatti et al., 1991).

Another possibility to explain the enhancement of tube formation by the antibodies is that the binding of the anti-integrin antibodies to their antigen may simply limit the number of receptors available for cell-matrix interactions thereby resulting in enhanced motility of the cell within the matrix. Indeed, an alteration in the adhesivity of EC for different matrices via changes in the level of expression of the integrins, $\alpha_1\beta_1$ and $\alpha_v\beta_3$, can be achieved by cytokines such as tumor necrosis factor and interferon- γ (Defilippi et al., 1991a,b). The anti-integrin antibodies may also limit the number of focal contacts which can form an important function mediated through the β subunit (Solowska et al., 1989), reducing adhesion, and enhancing the lateral mobility of the integrins within the cell membrane. This lateral mobility of integrins is known to be important for cell movement (Duband et al., 1988). Alternately, the limitation in the available number of functional $\alpha_2\beta_1$ or $\alpha_v\beta_3$ molecules may redirect the cell to use other integrin molecules. It is interesting to note that RMAC11 or AK7 cannot totally inhibit HUVEC binding to collagen. Anti- β_1 antibodies further inhibit this adhesion suggesting that other β_1 integrins are involved. $\alpha_1\beta_1$ and $\alpha_3\beta_1$ are able to mediate adhesion to collagen at least in some cells (Defilippi et al., 1991a; Hemler et al., 1990). Whether these integrins can participate in angiogenesis remains to be determined. The use of alternate matrix receptors may induce a different set of signals which in our system is manifested in enhanced capillary formation. Using chimeric constructs transfected into RD cells, Chan et al. (Chan et al., 1992) have shown that the cytoplasmic domains of the integrin receptors can mediate different signals irrespective of the ligand-binding event.

Angiogenesis is clearly a complex event that can be regulated at multiple levels. In this paper we have demonstrated that the extent of capillary tube formation can be enhanced by the use of anti-integrin antibodies specific for a given receptor-ligand system which inhibit cell-matrix adhesion.

These antibodies have profound effects on cell shape, adhesion, proliferation, and subsequent cell differentiation. A corollary of the work presented here suggests that anti-integrin antibodies which promote cell adhesion to the ECM will actually inhibit *in vitro* angiogenesis. Thus regulation of angiogenesis may be mediated through alteration in the matrix (as has been shown previously), or as our results suggest, by alteration in the function of matrix-adhesion receptors on the EC.

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