[URE3] strains and that from [ure3] cells. It is also vital to show that the isolated Ure2p can transmit the [URE3] character. But such data in the scrapie field has not quelled controversy concerning the nature of the infectious entity because the low ratio of infectious units to molecules makes ruling out a nucleic acid component difficult.

If, as suggested here, prions are a more general phenomenon than the essentially single mammalian case, are there not other phenomena which could be explained in this way? [PSI] is a non-Mendelian genetic element of *S. cerevisiae* discovered by Cox by its enhancement of ochre suppression [reviewed in (*18, 19*)]. [PSI] is reversibly curable (like [URE3]). The *PNM2* gene (PSI no more), necessary for propagation of [PSI], is identical to *sup35/sal3/SUP2/suf12* and is intimately involved in translational fidelity, and many *pnm2* mutants have a [PSI]-like phenotype (like *ure2* mutants having a [URE3]-like phenotype) (*18, 19*). Finally, *PNM2* on a high-copy plasmid results in frequent de novo generation of [PSI] (*20*). The logical parallels with [URE3] and *URE2* make a compelling case that [PSI] is a prion form of the *PNM2* protein.

REFERENCES AND NOTES

1. D. C. Gajdusek, *Eur. J. Epidemiol.* **7**, 567 (1991); S. B. Prusiner, *Science* **252**, 1515 (1991); C. Weissmann, *Nature* **352**, 679 (1991); J. S. Griffith, *ibid.* **215**, 1043 (1967); S. B. Prusiner, *Science* **216**, 136 (1982).
2. H. Bueler *et al.*, *Cell* **73**, 1339 (1993); S. B. Prusiner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10608 (1993).
3. R. B. Wickner, *Annu. Rev. Microbiol.* **46**, 347 (1992).
4. Nomenclature: For *URE2*, as for all chromosomal genes, the dominant allele is shown in capital italics. The wild-type allele is dominant and the mutant, *ure2*, is recessive. Ure2p means the protein product of *URE2*. The brackets in [URE3] indicate that it is a non-Mendelian element and the capitals that it is dominant, although [URE3] strains are isolated as "mutants." The wild-type (recessive) state is [ure3]. Aspartate transcarbamylase is encoded by *URA2*, so the ability of *ura2* mutants to grow on ureidosuccinate in the presence of ammonia is used to assess *URE2* and [URE3].
5. R. Drillien and F. Lacroute, *J. Bacteriol.* **109**, 203 (1972).
6. F. Lacroute, *ibid.* **106**, 519 (1971).
7. B. Magasanik, in *The Molecular and Cellular Biology of the Yeast* Saccharomyces, E. W. Jones, J. R. Pringle, J. R. Broach, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992), vol. 2, pp. 283–317.
8. Assay of [URE3] and genetic manipulations: Minimal medium (SD) and rich medium (YPAD) were as described (*21*). SGal is SD with 2% galactose in place of dextrose. Strains carrying the [URE3] element or a mutation in the chromosomal *URE2* gene are able to take up ureidosuccinate in the presence of either ammonium ion or glutamine to supply the defect in a *ura2* mutant. The assay medium routinely used was SD to which was added ureidosuccinate (100 μg/ml) and any amino acids required by the strain to be tested. This medium had ammonium as the nitrogen source. Transfer of [URE3] by cytoplasmic mixing (cytoduction) was carried out as described (*22*). The designation ρ^0 means that the strain lacks mitochondrial DNA. Strains were cured of [URE3] by streaking for single colonies on YPAD plates containing 5 mM guanidine HCl. Single colonies were tested for utilization of ureidosuccinate. Generally all were unable to do so.
9. M. Aigle and F. Lacroute, *Mol. Gen. Genet.* **136**, 327 (1975).
10. M. Aigle, personal communication.
11. F. Foury, *J. Biol. Chem.* **264**, 20552 (1989).
12. Plasmid construction and antibodies: YEp351-URE2 was constructed by the insertion of the 2.7-kb Sal I–Bgl II fragment of p1-XS carrying *URE2* (*16*) into YEp351 (*23*) cut with Bam HI and Sal I. The same *URE2* fragment inserted into Sal I–Bam HI–cut pRS315 (*24*) produced p532, which was mutagenized (*25*) with the oligonucleotide UG = 5′-GACGGAGCGCATTGGATCCATTCGACACTTGGTTG-3′ to place a Bam HI site just 3′ of the initiator AUG of *URE2*. The 1.2-kb Bam HI–Eco RI fragment of the resulting plasmid, p578, carrying most of *URE2* was inserted into pGEX-2T (Pharmacia) cut with the same two enzymes to produce an in-frame fusion with GST (p580). This fusion protein was used to immunize rabbits (Berkeley Antibody Company). To place *URE2* under galactose control, plasmid YEp351G with the 0.9-kb *GAL1* promoter inserted between the Eco RI and Bam HI sites of YEp351 (*26*) was used. The plasmid p532 was mutagenized with the oligonucleotide UB = 5′-GTTATTCATCATTTGGGATCCAACTTAATTTGCAGC-3′ which replaces bases 173 to 178 (just 5′ of the initiator AUG) with a Bam HI site, and YEp351G-URE2 was made by insertion of the 2.1-kb fragment from *URE2* base 179 to the Xba I site 3′ of the *URE2* open reading frame into YEp351G cut with Bam HI and Xba I. The bacteriophage f1 *ori* from pDM1 (*27*) on a 0.5-kb Sal I fragment was inserted into the Sal I site just 3′ of the *URE2* gene in YEp351G-URE2. The resulting plasmid, p649, was mutagenized with two oligonucleotides: 495 = 5′-GGTTGTTCTTGAATTCATTTTGTAATTCTG-3′, replacing amino acid codon 105 of *URE2* with UGA (opal) (p652 = YEp351G-URE2op), and 525 = 5′-CCTGTGAGAGAAAGCTTAATATCCCTCCAGT-3′, which replaces codon 115 with UAA (p655 = YEp351G-URE2oc). All mutations were confirmed by sequencing.
13. R. B. Wickner, unpublished data.
14. R. Drillien, M. Aigle, F. Lacroute, *Biochem. Biophys. Res. Comm.* **53**, 367 (1973).
15. W. E. Courchesne and B. Magasanik, *J. Bacteriol.* **170**, 708 (1988).
16. P. W. Coschigano and B. Magasanik, *Mol. Cell. Biol.* **11**, 822 (1991).
17. K. H. Kim and S. G. Rhee, *J. Biol. Chem.* **263**, 833 (1988).
18. B. S. Cox, M. F. Tuite, C. S. McLaughlin, *Yeast* **4**, 159 (1988).
19. B. S. Cox, in *The Early Days of Yeast Genetics*, M. N. Hall and P. Linder, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993), pp. 219–239.
20. Y. O. Chernoff, I. L. Derkach, S. G. Inge-Vechtomov, *Curr. Genet.* **24**, 268 (1993).
21. F. Sherman, in *Guide to Yeast Genetics and Molecular Biology*, C. Guthrie and G. R. Fink, Eds. (Academic Press, San Diego, 1991), vol. 194, pp. 3–21.
22. S. P. Ridley, S. S. Sommer, R. B. Wickner, *Mol. Cell. Biol.* **4**, 761 (1984).
23. J. E. Hill *et al.*, *Yeast* **2**, 163 (1986).
24. R. S. Sikorski and P. Hieter, *Genetics* **122**, 19 (1989).
25. T. A. Kunkel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488 (1985).
26. B. Benton *et al.*, *Mol. Cell. Biol.* **10**, 353 (1990).
27. D. A. Mead, E. S. Skorupa, B. Kemper, *Nucleic Acids Res.* **13**, 1103 (1985).

28. I thank M. Aigle and F. Lacroute for providing strains and information about [URE3] and P. Coschigano and B. Magasanik for strains, clones, and information about *URE2*.

16 December 1993; accepted 28 February 1994

Requirement of Vascular Integrin $\alpha_v\beta_3$ for Angiogenesis

Peter C. Brooks, Richard A. F. Clark, David A. Cheresh*

Angiogenesis depends on the adhesive interactions of vascular cells. The adhesion receptor integrin $\alpha_v\beta_3$ was identified as a marker of angiogenic vascular tissue. Integrin $\alpha_v\beta_3$ was expressed on blood vessels in human wound granulation tissue but not in normal skin, and it showed a fourfold increase in expression during angiogenesis on the chick chorioallantoic membrane. In the latter assay, a monoclonal antibody to $\alpha_v\beta_3$ blocked angiogenesis induced by basic fibroblast growth factor, tumor necrosis factor–α, and human melanoma fragments but had no effect on preexisting vessels. These findings suggest that $\alpha_v\beta_3$ may be a useful therapeutic target for diseases characterized by neovascularization.

The growth of new blood vessels, or angiogenesis, plays a key role in development, wound repair, and inflammation. This process also contributes to pathological conditions such as diabetic retinopathy, rheumatoid arthritis, and cancer (*1–6*), and there has been much interest in developing therapeutic agents that inhibit angiogenesis in these contexts. Identification of the molecules that regulate angiogenesis is critical to the success of such targeted therapies.

Angiogenesis is characterized by the invasion, migration, and proliferation of smooth muscle and endothelial cells; thus, it seems likely that vascular cell adhesion molecules contribute to its regulation (*2*,

P. C. Brooks and D. A. Cheresh, Department of Immunology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA.
R. A. F. Clark, Department of Dermatology, School of Medicine, State University of New York, Stony Brook, NY 11794–0001, USA.

*To whom correspondence should be addressed.

7, 8). During wound healing, the basement membrane zones of blood vessels express several adhesive proteins, including von Willebrand factor, fibronectin, and fibrin. In addition, several members of the integrin family of adhesion receptors are expressed on the surface of cultured smooth muscle and endothelial cells (9–11). Among these integrins is $\alpha_v\beta_3$, the endothelial cell receptor for von Willebrand factor, fibrinogen (fibrin), and fibronectin (9). This integrin initiates a calcium-dependent signaling pathway leading to endothelial cell migration and, therefore, appears to play a fundamental role in vascular cell biology (12).

To investigate the expression of $\alpha_v\beta_3$ during angiogenesis, we stained human wound granulation tissue or adjacent normal skin with antibodies specific for either β_3 integrins ($\alpha_v\beta_3$ or $\alpha_{IIb}\beta_3$) or the β_1 subfamily of integrins. The $\alpha_v\beta_3$ integrin was abundantly expressed on blood vessels in granulation tissue but was not detectable in the dermis and epithelium of normal skin from the same donor (Fig. 1). In contrast, β_1 integrins were abundantly expressed on blood vessels and stromal cells in both normal skin and granulation tissue and, as previously shown (13), on the basal cells within the epithelium. The same tissues were examined for the presence of the ligands for the β_3 and β_1 integrins, von Willebrand factor and laminin, respectively. Von Willebrand factor localized to the blood vessels in normal skin and granulation tissue, whereas laminin localized to all blood vessels as well as the epithelial basement membrane (14).

Angiogenesis can be induced on the chick chorioallantoic membrane (CAM) in response to specific cytokines or tumor fragments (15, 16). This assay was used to examine the effect of two potent angiogenesis inducers, basic fibroblast growth factor (bFGF) and tumor necrosis factor–α (TNF-α), on the expression of β_1 and β_3 integrins. Filter disks impregnated with either cytokine and placed on CAMs from 10-day embryos were found to promote local angiogenesis after 72 hours (14). Cryostat sections of control or bFGF-treated CAMs were stained with monoclonal antibodies (mAbs) CSAT [directed to chick β_1 integrins (anti-β_1) (17)] or LM609 [directed to chick (18) and human (9) $\alpha_v\beta_3$ (anti-$\alpha_v\beta_3$)]. Integrin β_1 was readily detectable on all control (Fig. 2A, top) and stimulated blood vessels (19), whereas $\alpha_v\beta_3$ was detectable on 75 to 80% of the vessels on the bFGF-treated CAMs (Fig. 2A, bottom) but not on untreated CAMs (Fig. 2A, center). These findings indicate that in both human and chick, blood vessels involved in angiogenesis have enhanced expression of $\alpha_v\beta_3$. Consistent with this result, expression of $\alpha_v\beta_3$ on cultured endothelial cells can be induced by various cytokines in vitro (10, 20, 21).

We next quantified the relative expression of $\alpha_v\beta_3$ and β_1 integrins during bFGF-induced angiogenesis by laser confocal image analysis of the CAM cryostat sections. Expression of β_1 was high in both untreated and bFGF-treated tissue; in contrast, $\alpha_v\beta_3$ expression in bFGF-treated tissues was four times that of untreated tissue ($P < 0.0001$) (Fig. 2B).

To determine whether $\alpha_v\beta_3$ plays an active role in angiogenesis, we saturated filter disks placed on CAMs with bFGF or TNF-α and then added anti-$\alpha_v\beta_3$, anti-β_1, or mAb P3G2 (anti-$\alpha_v\beta_5$). The anti-$\alpha_v\beta_3$ blocked bFGF-induced angiogenesis, whereas anti-β_1 and -$\alpha_v\beta_5$ had no effect. Identical results were obtained when angiogenesis was induced with TNF-α (14). To examine the effects of these same antibodies on preexisting blood vessels, we placed filter disks saturated with mAbs but without cytokine on vascularized regions of CAMs from 10-day embryos. None of the three mAbs affected preexisting vessels, as assessed by visualization under a stereomicroscope (14). Thus, anti-$\alpha_v\beta_3$ appears to act selectively on new blood vessel growth.

To determine whether $\alpha_v\beta_3$ participates in embryonic angiogenesis, we examined the effect of anti-$\alpha_v\beta_3$ on de novo growth of blood vessels on CAMs from younger (6-day) embryos, a stage marked by active

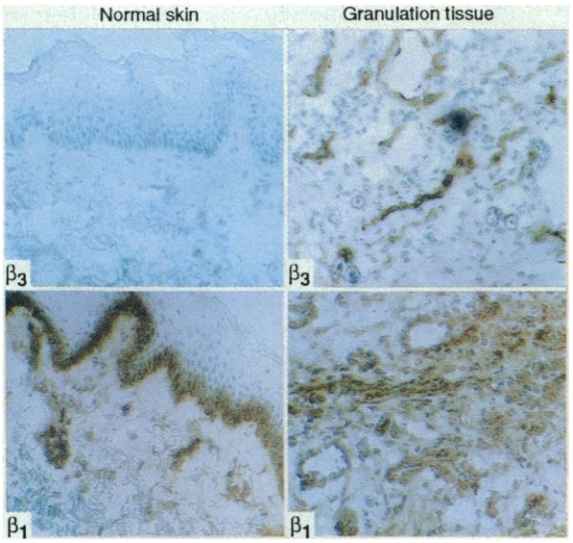

Fig. 1. Immunoperoxidase staining of normal human skin and wound granulation tissue. Monoclonal antibodies AP3 and LM534, directed to β_3 and β_1 integrins, respectively, were used for immunohistochemical analysis of frozen sections (25). Experiments with tissue from four human donors yielded identical results. Magnification, ×121.

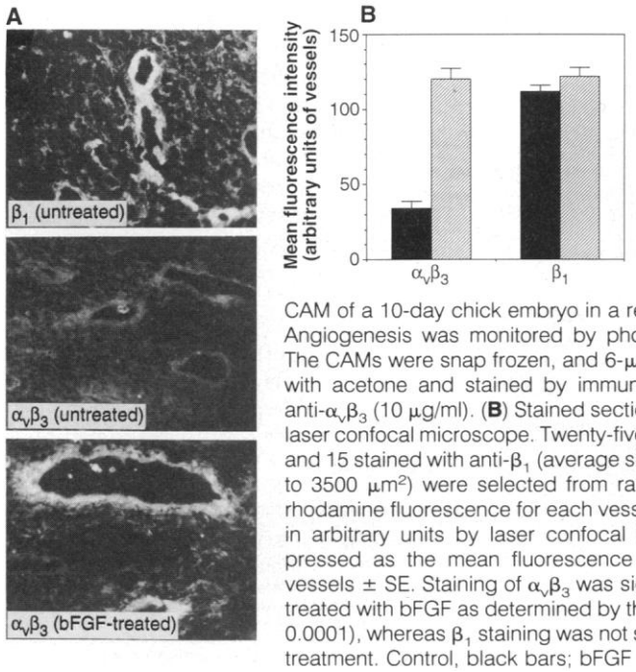

Fig. 2. Enhanced expression of $\alpha_v\beta_3$ during cytokine-induced angiogenesis on the chick CAM. (**A**) Angiogenesis was induced by a 5 mm by 5 mm Whatman filter disk saturated with Hanks balanced salt solution (HBSS) (untreated) or HBSS containing recombinant bFGF (150 ng/ml) (bFGF-treated) and placed on the CAM of a 10-day chick embryo in a region devoid of blood vessels. Angiogenesis was monitored by photomicroscopy after 72 hours. The CAMs were snap frozen, and 6-μm cryostat sections were fixed with acetone and stained by immunofluorescence with anti-β_1 or anti-$\alpha_v\beta_3$ (10 μg/ml). (**B**) Stained sections were analyzed with a Zeiss laser confocal microscope. Twenty-five vessels stained with anti-$\alpha_v\beta_3$ and 15 stained with anti-β_1 (average size was ~1200 μm^2; range 350 to 3500 μm^2) were selected from random fields, and the average rhodamine fluorescence for each vessel per unit area was measured in arbitrary units by laser confocal image analysis. Data are expressed as the mean fluorescence intensity in arbitrary units of vessels ± SE. Staining of $\alpha_v\beta_3$ was significantly enhanced on CAMs treated with bFGF as determined by the Wilcoxon rank sum test ($P < 0.0001$), whereas β_1 staining was not significantly different with bFGF treatment. Control, black bars; bFGF treatment, hatched bars.

neovascularization (*22*). The anti-$\alpha_v\beta_3$, but not anti-β_1 or anti-$\alpha_v\beta_5$, prevented vascular growth under these conditions (Fig. 3B); thus, $\alpha_v\beta_3$ appears to play a role in embryonic neovascularization.

We also investigated the role of $\alpha_v\beta_3$ in tumor-induced angiogenesis. As an inducer, we used $\alpha_v\beta_3$-negative human M21-L melanoma fragments previously grown and isolated from the CAM of a 17-day chick embryo (*23*). These fragments induced extensive neovascularization in the presence of buffer alone, or anti-β_1 or anti-$\alpha_v\beta_5$. In contrast, anti-$\alpha_v\beta_3$ abolished the infiltration of most vessels into the tumor mass and surrounding CAM (Fig. 4A). Quantitative analysis (Fig. 4B) revealed that one-third the number of vessels entered tumors treated with anti-$\alpha_v\beta_3$ compared with tumors treated with buffer or the other mAbs ($P < 0.0001$). The fact that M21-L tumors do not express $\alpha_v\beta_3$ suggests that anti-$\alpha_v\beta_3$ inhibits angiogenesis by directly affecting blood vessels rather than the tumor cells.

Our results demonstrate that integrin $\alpha_v\beta_3$ plays a key role in angiogenesis induced by a variety of stimuli. The finding that an antibody to $\alpha_v\beta_3$ inhibits the growth of new vessels without perturbing preexisting vessels is perhaps not surprising, given that this integrin appears to be selectively expressed on growing vessels. In fact, recent clinical trials have revealed that a function-blocking antibody directed to the β_3 integrin subunit, reactive with $\alpha_v\beta_3$ as well as platelet $\alpha_{IIb}\beta_3$, significantly reduced symptoms of restenosis in patients who had undergone angioplasty (*24*). Together, these data suggest that integrin $\alpha_v\beta_3$ will be a useful therapeutic target for diseases characterized by neovascularization.

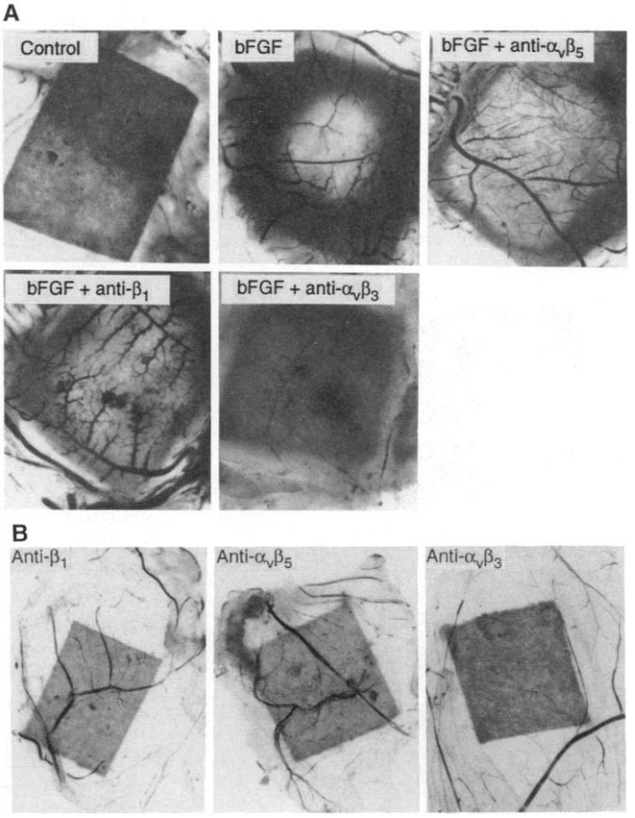

Fig. 3. Inhibition of cytokine-induced and embryonic angiogenesis by anti-$\alpha_v\beta_3$. (**A**) Angiogenesis was induced on CAMs from 10-day chick embryos by filter disks saturated with bFGF. Disks were then treated at 0, 24, and 48 hours with 50 μl of HBSS containing 25 μg of mAb. At 72 hours, CAMs were harvested and analyzed under an Olympus stereomicroscope by two observers in a double-blind fashion. Angiogenesis inhibition was considered significant when CAMs exhibited >50% reduction in blood vessel infiltration of the CAM directly under the disk. Experiments were repeated four times per antibody, with six to seven embryos per condition. About 75% of the CAMs treated with anti-$\alpha_v\beta_3$ exhibited >50% inhibition of angiogenesis, and many of these appeared devoid of vessel infiltration. In contrast, the buffer control and disks treated with anti-β_1 and anti-$\alpha_v\beta_5$ consistently showed extensive vascularization. (**B**) Disks saturated with mAbs were placed on CAMs of 6-day-old embryos in the absence of cytokines. After 72 hours, CAMs were resected and photographed. Each experiment included six embryos per group and was repeated twice.

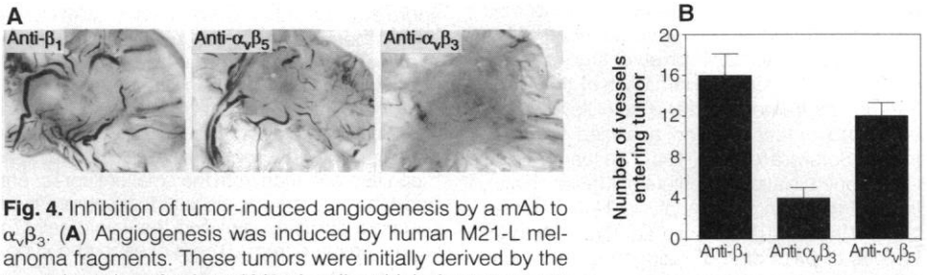

Fig. 4. Inhibition of tumor-induced angiogenesis by a mAb to $\alpha_v\beta_3$. (**A**) Angiogenesis was induced by human M21-L melanoma fragments. These tumors were initially derived by the transplantation of cultured M21-L cells, which do not express integrin $\alpha_v\beta_3$ (*26*), onto the CAMs of 10-day-old chick embryos (*23*). After 7 days, tumors were resected, sliced into 20-mg fragments, and placed on CAMs from 10-day-old embryos in regions devoid of blood vessels. Antibodies (25 μg per 50 μl) were applied topically to tumor fragments, and after 3 days the tumors and surrounding CAMs were resected and photographed. Experiments were repeated twice per antibody. (**B**) Blood vessels entering the tumor within the focal plane of the CAM were counted under a stereomicroscope by two observers in a double-blind fashion. Each bar represents the mean number of vessels ± SE from 12 CAMs in each group. There were significantly fewer vessels entering tumors treated with anti-$\alpha_v\beta_3$ compared with those treated with mAbs to $\alpha_v\beta_5$ or β_1 ($P < 0.0001$) as determined by Wilcoxon rank sum test.

REFERENCES AND NOTES

1. H. F. Dvorak, *N. Engl. J. Med.* **315**, 1650 (1986).
2. R. A. F. Clark *et al.*, *J. Exp. Med.* **156**, 646 (1982).
3. J. Folkman, *Semin. Cancer Biol. Ser.* **3**, 65 (1992).
4. N. Weidner *et al.*, *J. Natl. Cancer Inst.* **84**, 1875 (1992).
5. D. Ingber *et al.*, *Nature* **348**, 555 (1990).
6. M. Yamaoka *et al.*, *Cancer Res.* **53**, 4262 (1993).
7. D. Ingber, *J. Cell. Biochem.* **47**, 236 (1991).
8. M. Nguyen, N. A. Strubel, J. Bischoff, *Nature* **365**, 267 (1993).
9. D. A. Cheresh, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6471 (1987).
10. M. F. Janat, W. S. Argraves, G. Liau, *J. Cell. Physiol.* **151**, 588 (1992).
11. Y. F. Cheng and R. H. Kramer, *ibid.* **139**, 275 (1989).
12. D. I. Leavesley, M. A. Schwartz, M. Rosenfeld, D. A. Cheresh, *J. Cell Biol.* **121**, 163 (1993).
13. J. C. Adams and F. M. Watt, *Cell* **63**, 425 (1991).
14. P. C. Brooks and D. A. Cheresh, unpublished data.
15. S. I. Leibovich *et al.*, *Nature* **329**, 630 (1987).
16. D. H. Ausprunk, D. R. Knighton, J. Folkman, *Am. J. Pathol.* **79**, 597 (1975).
17. C. A. Buck, S. E. Duggan, A. F. Horwitz, *J. Cell Biol.* **103**, 2421 (1986).
18. J. Lawler, R. Weinstein, R. O. Hynes, *ibid.* **107**, 2351 (1988).
19. G. Klier and D. A. Cheresh, unpublished data.
20. J. Enenstein, N. S. Waleh, R. H. Kramer, *Exp. Cell Res.* **203**, 499 (1992).
21. R. A. Swerlick *et al.*, *J. Invest. Dermatol.* **99**, 715 (1993).
22. R. Auerbach, R. Alensman, L. Kubai, J. Folkman. *Int. J. Cancer* **15**, 241 (1975).
23. P. C. Brooks, J. Lin, D. L. French, J. P. Quigley, *J. Cell Biol.* **122**, 1351 (1993).
24. J. N. Woody, paper presented at the Commercial Prospects of Cell Adhesion meeting, San Diego, CA, 6 December 1993.
25. C. L. Gladson and D. A. Cheresh, *J. Clin. Invest.* **88**, 1924 (1991).
26. B. Felding-Habermann, B. M. Meuller, C. A. Romerdahl, D. A. Cheresh, *ibid.* **89**, 2018 (1992).

27. D.A.C. is supported by NIH (grants CA45726 and CA50286), the California Tobacco Related Disease Program, and the American Cancer Society. We thank P. Newman and C. Buck for antibodies AP3 and CSAT, respectively; R. Rudolph for the human tissues; and G. Klier for assistance with confocal analysis. This is The Scripps Research Institute manuscript No. 8412-IMM.

12 January 1994; accepted 14 March 1994