rylation state of newly synthesized Rb is a consequence of RA-induced growth arrest. This conclusion is supported by the observation that treatment of HL-60A cells with compounds such as TPA (*13*), DMSO, or sodium butyrate all resulted in a similar time course of the decrease of Rb phosphorylation (Fig. 3B, lanes 7 to 10), despite the fact that these compounds each affect a different cellular target. After 96 hours of treatment with RA, sodium butyrate, or DMSO, the cells exhibited an underphosphorylated Rb of 98 kD. As RNA blot (Northern) analysis revealed a normal sized mRNA (*13*), the origin of this truncated Rb could be due to the initiation of translation at an internal ATG codon of *RB1*. As this species of Rb is observed in cells treated with the various chemical inducers, its appearance may be related to the state of growth arrest rather than differentiation of the cells.

We have demonstrated that newly synthesized Rb in cells at the G_0 and G_1 phases is underphosphorylated and that newly synthesized Rb is phosphorylated at multiple sites only in cells at the G_1/S boundary and in the S phase. Our observations are consistent with the interpretation that the underphosphorylated form of Rb inhibits cell proliferation and that this form of Rb is rendered inactive by phosphorylation at one or more critical sites before the initiation of DNA synthesis. The SV40 large T antigen can only bind to, and presumably inactivate, the underphosphorylated form of Rb (*18*). Thus, the inactivation of Rb by phosphorylation may be an obligatory event for cells to traverse the G_1/S boundary. Control of cell proliferation may therefore be exerted at the level of Rb inactivation.

REFERENCES AND NOTES

1. M. Wahl *et al.*, *Mol. Cell. Biol.* **9**, 2934 (1989); J. Meisenhelder, P. Suth, S. G. Rhee, T. Hunter, *Cell* **57**, 1109 (1989).
2. M. Wahl, S. Nishibe, P. Suh, S. G. Rhee, G. Carpenter, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1568 (1989); B. Margolis *et al.*, *Cell* **57**, 1101 (1989).
3. T. Deuel, *Annu. Rev. Cell Biol.* **3**, 443 (1987).
4. B. H. Cochran, J. Zullo, I. M. Verma, C. D. Stiles, *Science* **226**, 1080 (1984); C. B. Thompson, P. B. Challoner, P. E. Neiman, M. Groudine, *Nature* **319**, 374 (1986); L. F. Lau and D. Nathans, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1182 (1987); K. Ryter, L. F. Lau, D. Nathans, *ibid.* **85**, 1487 (1988).
5. C. Schneider, R. M. King, L. Philipson, *Cell* **54**, 787 (1988); P. Bedard, Y. Yannoni, D. L. Simmons, R. L. Erikson, *Mol. Cell. Biol.* **9**, 1371 (1989).
6. S. H. Friend *et al.*, *Nature* **323**, 643 (1986); Y. K. T. Fung *et al.*, *Science* **236**, 1657 (1987); W.-H. Lee *et al.*, *ibid.* **235**, 1394 (1987).
7. J. W. Harbour *et al.*, *Science* **241**, 353 (1988); J. Yokota *et al.*, *Oncogene* **3**, 471 (1988).
8. A. T'Ang, J. M. Varley, S. Chakraborty, A. L. Murphree, Y.-K. T. Fung, *Science* **242**, 263 (1988).
9. P. Whyte *et al.*, *Nature* **334**, 124 (1988).
10. J. A. Decaprio *et al.*, *Cell* **54**, 275 (1988).
11. N. Dyson, P. M. Howley, K. Münger, E. Harlow, *Science* **243**, 934 (1989).
12. For preparation of antibodies against Rb, peptides synthesized according to the protein sequence deduced from the *RB1* cDNA sequence were used to immunize young New Zealand rabbits. For Rb 1-AB 16, the peptide (P4) used was CEEIYLKNKDLDARLFLDHDK (amino acids 322 to 341) (*15*). For Rb 1-AB 20, the peptide (P5) used was CEGSNPPKPLKKLRFDIEGSDEAD (amino acids 864 to 886). For Rb 1-AB A1, the peptide (P2) used was CRMQKQKMNDSMDTSNKEEK (amino acids 910 to 928).
13. K. Mihara *et al.*, unpublished data.
14. W. H. Lee *et al.*, *Nature* **329**, 642 (1987); J. Varley *et al.*, *Oncogene* **4**, 721 (1989).
15. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
16. H.-J. Xu *et al.*, *Oncogene* **4**, 807 (1989).
17. A. Yen, V. Powers, J. Fishbaugh, *Leukemia Res.* **10**, 619 (1986).
18. J. Ludlow *et al.*, *Cell* **56**, 57 (1989).
19. B. A. Macher *et al.*, *Exp. Cell Res.* **117**, 95 (1978).
20. A. Yen, D. Brown, J. Fishbaugh, *ibid.* **168**, 247 (1987).
21. S. J. Collins, A. Bodner, R. Ting, R. C. Gallo, *Int. J. Cancer* **25**, 213 (1980).

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An Inducible Endothelial Cell Surface Glycoprotein Mediates Melanoma Adhesion

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Hematogenous metastasis requires the arrest and extravasation of blood-borne tumor cells, possibly involving direct adhesive interactions with vascular endothelium. Cytokine activation of cultured human endothelium increases adhesion of melanoma and carcinoma cell lines. An inducible 110-kD endothelial cell surface glycoprotein, designated INCAM-110, appears to mediate adhesion of melanoma cells. In addition, an inducible endothelial receptor for neutrophils, ELAM-1, supports the adhesion of a human colon carcinoma cell line. Thus, activation of vascular endothelium in vivo that results in increased expression of INCAM-110 and ELAM-1 may promote tumor cell adhesion and affect the incidence and distribution of metastases.

HEMATOGENOUS METASTASIS IS AN inefficient process, with most cancer cells failing to survive in the circulation [reviewed in (*1*)]. Endothelial injury or denudation enhances metastasis formation (*2*), suggesting that the lining of blood vessels may normally act as a barrier to tumor cell extravasation. However, tumor cells adhere focally to intact endothelium and subsequently transmigrate to the basal lamina, often after many hours (*3*). Characteristic patterns of metastatic spread have led to the suggestion that tumor cells preferentially interact with microvascular endothelium in particular organs or tissue sites (*4*). Activation of endothelium by cytokines increases the adhesion of human melanoma and carcinoma cells in vitro (*5–7*). Thus, tumor cell adhesion to the vessel wall may result from focal alterations in endothelial cells, perhaps involving the expression of specific cell surface molecules.

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Endothelial cell surface properties can be altered in response to cytokines, bacterial endotoxin, and coagulation factors (*8*). Interleukin-1 (IL-1), tumor necrosis factor (TNF), and endotoxin increase leukocyte adhesion through biosynthesis and expression of two cell surface glycoproteins, endothelial leukocyte adhesion molecule 1 (ELAM-1) (*9*) and intercellular adhesion molecule 1 (ICAM-1) (*10, 11*). Monoclonal antibody (MAb) studies did not demonstrate a role for ELAM-1 (*5*) or ICAM-1 (*5–7*) in melanoma cell adhesion.

To identify endothelial surface structures involved in the adhesion of melanoma cells, MAbs were generated to TNF-stimulated human endothelial cells (HECs) from umbilical vein (*12*). The MAb E1/6 recognized an inducible HEC surface antigen whose expression paralleled adhesiveness for melanoma cells. Continuous exposure of HEC monolayers to recombinant TNF resulted in increased melanoma adhesion (Fig. 1A), and increased expression of E1/6 antigen (Fig. 1B) that was detectable at 2 hours, maximal at 6 to 8 hours (an increase of 13.6 ± 1.9 times in MAb binding, mean

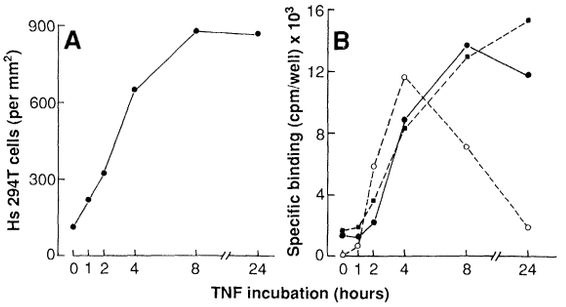

Fig. 1. Comparison of melanoma cell adhesion and HEC surface expression of E1/6 antigen, ELAM-1, and ICAM-1. (**A**) Serially passaged HECs [subculture 2 (*5*)] grown in microtiter wells were treated with TNF (200 U/ml, gift of Biogen) for the indicated time periods. The monolayers were then washed, metabolically labeled human melanoma cells (Hs 294T, obtained from ATCC), were added (5×10^4/well), and a 30-min adhesion assay was performed (*5*). Data represent means of quadruplicate microtiter wells. (**B**) HEC monolayers were treated with TNF (200 U/ml), and exposed to MAbs E1/6, H18/7 (anti–ELAM-1), or LB-2 (anti–ICAM-1) (4°C, 1 hour). MAb binding to intact HEC monolayers was assessed with ^{125}I-labeled F(ab')$_2$ fragments of antibody to mouse immunoglobulin (Ig) (*9*). Specific counts were determined by subtracting counts per minute determined with irrelevant, isotype-matched MAbs (generally less than 10^3 cpm). Data points represent the means of quadruplicate microtiter wells. One of three similar experiments is depicted.

± SEM, six experiments), and maintained through 48 hours. Comparable results were observed with IL-1– or endotoxin-treated HEC monolayers. The kinetics of E1/6 antigen expression on TNF-activated HECs differed significantly from that of ELAM-1, which was maximal at 4 hours and declined toward basal levels by 24 hours (*9*), but was similar to that of ICAM-1 (*11, 13*) (Fig. 1B). Low levels of E1/6 antigen and ICAM-1, but not ELAM-1, were expressed on unstimulated HECs. The E1/6 antigen was not detected on the human melanoma cell lines SK-MEL-24 or Hs 294T by indirect radioimmunoassay, immunostaining, or flow cytometry.

Adhesion of SK-MEL-24 cells to TNF-activated HEC monolayers was blocked by MAb E1/6, whereas MAb H18/7 (anti–ELAM-1) and MAb LB-2 (anti–ICAM-1) had no significant effect (Fig. 2). Similarly, the adhesion of three human melanoma cell lines (SK-MEL-24, Hs 294T, and HT-144) to IL-1– or endotoxin-stimulated HECs was inhibited by MAb E1/6 (Table 1). Exposure of melanoma cells to MAb E1/6 before assays (followed by washing) did not block adhesion, whereas similar treatment of HEC monolayers was effective (34 ± 7% inhibition of Hs 294T cell adhesion, mean ± SEM, two experiments). The MAb E1/6 did not inhibit adhesion of Hs 294T cells to subendothelial matrix exposed after nonenzymatic removal of HECs (2 m*M* EDTA).

Murine B16-F10 melanoma cells (*14*) also exhibited increased adhesion to TNF-activated HEC monolayers (an increase of 3.6 ± 1.0 times, five experiments). The MAb E1/6 inhibited B16 melanoma cell adhesion to activated HECs (Table 1), suggesting that a mechanism for melanoma cell binding to the endothelial structure identified by MAb E1/6 may be conserved across species lines. Anti–ELAM-1 and anti–ICAM-1 MAbs had no effect on B16-F10 cell adhesion.

The human colon carcinoma cell line HT-29 also adhered to TNF-activated HEC monolayers more than to unstimulated monolayers (an increase of 2.5 ± 0.4 times, mean ± SEM, four experiments) (*6*). In contrast to its effect on melanoma cells, MAb E1/6 did not inhibit HT-29 carcinoma cell adhesion to HECs (Table 1). However, a MAb directed against ELAM-1 (H18/7) was effective (59 ± 10% inhibition, mean ± SEM, four experiments). These data are consistent with the relatively transient effect of endothelial activation on HT-29 cell adhesion [peak at 4 hours, decline by 24 hours (*6*)], which parallels the cell surface expression of ELAM-1 (Fig. 1B).

In immunoprecipitation studies, MAb E1/6 reacted with polypeptide species of 110 kD and 95 kD derived from total cell extracts of biosynthetically labeled, TNF-stimulated HECs (Fig. 3A). A single band corresponding to a polypeptide of 76 kD was observed after treatment of the HEC precipitate with *N*-glycosidase F to remove N-linked carbohydrates (Fig. 3A). Biosynthetic studies ("pulse-chase") revealed that label first appeared in the 95-kD species and was subsequently detected in the 110-kD species (Fig. 3B), concurrent with a decrease in intensity of the 95-kD band. Transiently labeled higher molecular size species (>150 kD) observed in these studies most likely represent nonspecific background polypeptides, since they were also observed in studies with two other MAbs. Taken together, these data suggest that the 95-kD and 110-kD species differ in glycosylation.

Expression of E1/6 antigen in human tissues was examined by immunoperoxidase techniques (*15*). In six of ten specimens of human lung, MAb E1/6 bound to vascular endothelial cells in a proportion of small blood vessels (typically 10 to 30%). The most extensive endothelial staining (approximately 50% of vessels) was observed in a

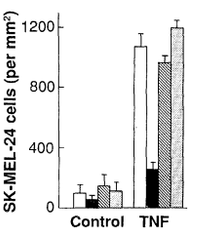

Fig. 2. Effect of MAbs E1/6, H18/7 (anti–ELAM-1), and LB-2 (anti–ICAM-1) on SK-MEL-24 cell adhesion to HECs. Unstimulated and TNF-stimulated (200 U/ml, 6 hours) HEC monolayers were incubated in medium containing no MAb (white bars), MAb E1/6 (culture supernatant) (black bars), MAb H18/7 (10 μg of ascites protein per milliliter) (hatched bars), or MAb LB-2 (10 μg/ml) (dotted bars) for 30 min at 37°C. Radiolabeled SK-MEL-24 cells (5×10^4 per microtiter well) were added without removal of MAbs, and incubated (25°C) for an additional 30 min to allow adhesion. Plates were sealed with acetate tape covers, inverted, and centrifuged to remove nonadherent cells (*5*). Bars represent means ± SEM of quadruplicate microtiter wells. In separate studies, culture supernatant of the parent myeloma cell line (NS-1) had no effect on melanoma cell adhesion. MAb 4/45 (IgG1), which binds equally to unstimulated and cytokine-stimulated HECs, as well as to melanoma cells, also had no effect.

Table 1. Percent inhibition of tumor cell-endothelial adhesion by MAb El/6. Human umbilical vein endothelial cell monolayers were incubated for 6 to 8 hours in M199 with 20% FBS alone, or supplemented with IL-1 (5 U/ml), TNF (200 U/ml), or endotoxin (1 μg/ml). HECs were then washed and incubated for 30 min in RPMI with 10% horse serum, E1/6 culture supernatant, or with control MAb before the addition of tumor cells (5×10^4 per microtiter well). Data are represented as percent decrease in adhesion of tumor cells to HECs exposed to MAb E1/6, compared to no MAb (means ± SEM of two to six experiments, each performed with quadruplicate microtiter wells). Control MAbs H4/45 and E1/1 had no significant effect on tumor cell adhesion to unstimulated or stimulated HECs. In the absence of MAb, tumor cell adhesion to IL-1–, TNF-, and endotoxin-treated HECs ranged between 500 and 1500 cells/mm^2, an increase of two to five times over control monolayers. ND, not detected.

Cell line	Decrease in adhesion (%)		
	IL-1	TNF	Endotoxin
Human melanoma SK-MEL-24	67 ± 9	71 ± 7	70 ± 3
Human melanoma Hs 294T	63 ± 7	60 ± 5	60 ± 12
Human melanoma HT-144	45 ± 1	50 ± 6	ND
Murine melanoma B16-F10	49 ± 11	46 ± 10	37 ± 15
Human carcinoma HT-29	−2 ± 18	8 ± 3	ND

lung specimen in which ELAM-1 antigen, a marker of endothelial activation (*16*), was also expressed. Small vessels of tonsil ($n = 2$), and to a lesser extent peripheral lymph node ($n = 4$), also expressed E1/6 antigen focally. Although E1/6 antigen was most often found in endothelium of postcapillary venules and veins, it was also observed in arterial endothelium in lung and lymphoid tissue. Some high endothelial venules bound MAb E1/6, but these specialized postcapillary vessels of lymphoid tissue were not selectively marked. Initial evidence suggests that certain disease processes associated with endothelial activation result in the upregulation of E1/6 antigen (*17*). In addition, MAb E1/6 bound to certain nonendothelial cell types, notably cell populations of dendritic morphology within organized lymphoid tissues.

The biochemical characteristics, patterns of expression, and functional properties of E1/6 protein distinguish it from previously described endothelial molecules involved in cell-cell adhesion, including the cytokine-inducible glycoproteins ELAM-1 (*9*) and ICAM-1 (*10, 11, 18, 19*), and the vascular addressins (*20*). We propose to designate this structure "inducible cell adhesion molecule 110" (INCAM-110) (*21*). ELAM-1, an inducible endothelial receptor for neutrophils (*9*), also supported the adhesion of a colon carcinoma cell line, suggesting that tumor cells may recognize endothelial cell surface molecules that function normally in the adhesion of blood leukocytes. Interestingly, initial studies indicate that INCAM-110 functions as an endothelial-lymphocyte adhesion molecule. The putative tumor (and leukocyte) cell surface ligands for INCAM-110 and ELAM-1 have not yet been defined. However, the presence of an NH_2-terminal lectinlike domain in ELAM-1 (*9*) suggests that tumor cell and leukocyte surface carbohydrates may be involved in its adhesive interactions.

Tumor cell extravasation may involve sequential adhesive interactions with vascular endothelium (*4*) and subendothelial matrix (*22*). INCAM-110 and ELAM-1 provide potential mechanisms for direct adhesion of tumor cells to endothelial cells. In a patient with cancer, endothelial activation with increased expression of these molecules could result from host cell production of TNF/cachectin (*23*) or from concurrent disease processes [for example, infection or sepsis (*8, 24*)]. Tumor cells themselves may also produce cytokines (*25*), which raises the possibility that malignant cells arrested within a vessel could promote surface alterations in adjacent endothelium.

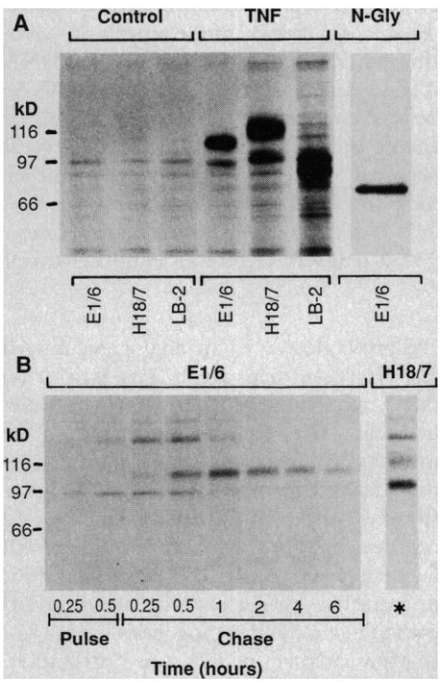

Fig. 3. (**A**) Autoradiogram of SDS-polyacrylamide gel comparing biosynthetically labeled ([^{35}S]cysteine/methionine) proteins precipitated from unstimulated and TNF-stimulated HEC with MAbs E1/6, H18/7, and LB-2. HEC monolayers were incubated in the presence of L-[^{35}S]cysteine and L-[^{35}S]methionine (Du Pont Biotechnology Systems) for 5 hours with or without TNF (*9*), and lysed with 2 m*M* tris-HCl plus 2% Nonidet P-40 and 1 m*M* phenylmethylsulfonyl fluoride (*p*H 7.4) for 30 min at 4°C. For immunoprecipitation, HEC lysates (50 to 100 μl) were incubated for 12 to 16 hours at 4°C with MAbs, and subsequently with anti-mouse immunoglobulin coupled to Sepharose-4B (2 hours, 4°C, Cooper Biomedicals, pretreated with lysates of unlabeled, unstimulated HECs to diminish nonspecific adherence of labeled proteins). Beads were washed and boiled in buffer containing 0.5 m*M* SDS and 2-mercaptoethanol (0.1 m*M*) (*9*). Immunoprecipitation samples from activated HECs were exposed to *N*-glycosidase F (*N*-Glycanase, Genzyme, 10 U/ml, 37°C, 18 hours) (last lane). Samples were separated through a 4 to 11% SDS-polyacrylamide gel. The autoradiograph shown is from one of four similar studies. MAb E1/l, which recognizes a constitutively expressed antigen, reacted with a 96-kD species in both control and activated endothelium. (**B**) Kinetic labeling ("pulse-chase") of E1/6 protein. HEC monolayers were treated for 1 hour with TNF (200 U/ml), washed, and incubated for an additional 30 min in RPMI-1640 without L-cysteine and L-methionine, supplemented with 10% dialyzed fetal bovine serum (FBS) and TNF. L-[^{35}S]Cysteine and L-[^{35}S]methionine were then added for 15 or 30 min. Certain samples were lysed immediately (pulse), while others (after 30-min labeling) were washed and placed in M199 with 20% FBS with TNF for various times (chase) before lysis and gel electrophoresis. Transiently labeled species of molecular size >150 kD were observed in pulse-chase studies with MAb H18/7 (anti–ELAM-1) (*shown at 0.25 hour chase) and MAb E1/l.

REFERENCES AND NOTES

1. G. Poste and I. J. Fidler, *Nature* **283**, 139 (1980); L. Weiss, F. W. Orr, K. V. Honn, *FASEB J.* **2**, 12 (1988).
2. I. Y. R. Adamson, F. W. Orr, L. Young, *J. Pathol.* **150**, 279 (1986); I. Y. R. Adamson, L. Young, F. W. Orr, *Lab. Invest.* **57**, 71 (1987).
3. S. Wood, Jr., *A. M. A. (Am. Med. Assoc.) Arch. Pathol.* **66**, 550 (1958); J. D. Crissman, J. Hatfield, M. Schaldenbrand, B. F. Sloane, K. V. Honn, *Lab. Invest.* **53**, 470 (1985).
4. L. Alby and R. Auerbach, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5739 (1984); R. Auerbach *et al.*, *Cancer Res.* **47**, 1492 (1987); G. L. Nicolson, *Biochim. Biophys. Acta.* **948**, 175 (1988); B. U. Pauli and C. Lee, *Lab. Invest.* **58**, 379 (1988).
5. G. E. Rice, M. A. Gimbrone, Jr., M. P. Bevilacqua, *Am. J. Pathol.* **133**, 204 (1988).
6. E. Dejana *et al.*, *J. Clin. Invest.* **82**, 1466 (1988).
7. D. Lauri *et al.*, *Lab. Invest.* **60**, 161 (1989).
8. R. S. Cotran and J. S. Pober, in *Endothelial Cell Biology*, N. Simionescu and M. Simionescu, Eds. (Plenum, New York, 1988), vol. 1, pp. 335–347; M. A. Gimbrone, Jr., and M. P. Bevilacqua, *ibid.*, pp. 255–274; D. M. Stern, D. A. Handley, P. P. Nawroth, *ibid.*, pp. 275–308; W. G. Owen, J. A. Goeken, P. Lollar, in *Vascular Endothelium in Hemostasis and Thrombosis*, M. A. Gimbrone, Jr., Ed. (Churchill Livingstone, London, 1986), pp. 57–69.
9. M. P. Bevilacqua, J. S. Pober, D. L. Mendrick, R. S. Cotran, M. A. Gimbrone, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **84**, 9238 (1987); M. P. Bevilacqua, S. Stengelin, M. A. Gimbrone, Jr., B. Seed, *Science* **243**, 1160 (1989).
10. M. L. Dustin and T. A. Springer, *J. Cell Biol.* **107**, 321 (1988); A. W. Boyd, S. O. Wawryk, G. F. Burns, J. V. Fecondo, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 3095 (1988); C. W. Smith *et al.*, *J. Clin. Invest.* **82**, 1746 (1988).
11. J. S. Pober *et al.*, *J. Immunol.* **137**, 1893 (1986).
12. BALB/c female mice were immunized with HECs that had been exposed to recombinant TNF-α (gift of Biogen Corp., Cambridge, MA) for 24 hours. Fusion of splenocytes with NS-1 myeloma cells was performed with polyethylene glycol (*9*), and a clone producing MAb E1/6 (IgG1) was selected on the basis of increased antibody binding to TNF-activated HECs in an indirect radioimmunoassay. Previously generated MAbs, including H18/7 (IgG2a) [which recognizes ELAM-1 (*9*)], MAb H4/45 (IgG1) and MAb E1/1 (IgG2a) [which recognize a 96-kD surface molecule constitutively expressed on HECs and mesenchymal cells (*9*)], MAb LB-2 (IgG2a) [which recognizes ICAM-1 (*19*)], and two nonbinding MAbs [K16/16 (IgG1) and E1A (IgG2a)] were used in comparative studies.
13. The potential reactivity of MAb E1/6 with ICAM-1 or ELAM-1 was further assessed with COS cells transfected with cDNAs encoding ELAM-1 or ICAM-1 (*9*). MAb El/6 did not react with COS cells expressing either recombinant ICAM-1 or recombinant ELAM-1 (as assessed by immunostaining).
14. I. J. Fidler, *Cancer Res.* **35**, 218 (1975).
15. Immunoperoxidase studies were done with cryostat sections of human tissues from the Department of Pathology, Brigham and Women's Hospital. Mouse MAbs were detected with biotinylated horse antibodies to mouse Ig (ABC, Vector) with diaminobenzidine as chromogen. Alternatively, peroxidase-conjugated rabbit antibodies to mouse Ig followed by peroxidase-conjugated swine antibodies to rabbit Ig (Dako) were used.
16. J. S. Pober *et al.*, *J. Immunol.* **136**, 1680 (1986); R. S. Cotran, M. A. Gimbrone, Jr., M. P. Bevilacqua, D. L. Mendrick, J. S. Pober, *J. Exp. Med.* **164**, 661 (1986); R. S. Cotran *et al.*, *J. Immunol.* **140**, 1883 (1988).
17. Little or no endothelial reactivity was observed in frozen sections of normal skin ($n = 4$), liver ($n = 3$), or colon ($n = 1$). However, strong staining of endothelium was observed in sites of inflammation in appendicitis and a delayed hypersensitivity reaction in the skin.
18. R. Rothlein, M. L. Dustin, S. D. Marlin, T. A. Springer, *J. Immunol.* **137**, 1270 (1986); M. L. Dustin, R. Rothlein, A. K. Bhan, C. A. Dinarello, T. A. Springer, *ibid.*, p. 245; D. Simmons, M. M.

Makgoba, B. Seed, *Nature* **331**, 624 (1988); D. E. Staunton, S. D. Marlin, C. Stratowa, M. L. Dustin, T. A. Springer, *Cell* **52**, 925 (1988); S. J. Mentzer, R. Rothlein, T. A. Springer, D. V. Faller, *J. Cell. Physiol.* **137**, 173 (1988).
19. M. Patarroyo, E. A. Clark, J. Prieto, C. Kantor, C. G. Gahmberg, *FEBS Lett.* **210**, 127 (1987); M. W. Makgoba *et al.*, *Nature* **331**, 86 (1988).
20. P. R. Streeter, E. L. Berg, B. T. N. Rouse, R. F. Bargatze, E. C. Butcher, *Nature* **331**, 41 (1988); N. W. Wu, S. Jalkanen, P. R. Streeter, E. C. Butcher, *J. Cell Biol.* **107**, 1845 (1988); P. R. Streeter, B. T. N. Rouse, E. C. Butcher, *ibid.*, p. 1853.
21. This molecule was previously referred to as "endothelial cell adhesion molecule" (ENDCAM) at the 1989 meeting of the Federation of American Societies for Experimental Biology in New Orleans, LA [*FASEB J.* **3**(4), A1052 (1989)]. Recent studies indicate that E1/6 antigen is also expressed on certain nonendothelial cell types, and we have chosen the designation "inducible cell adhesion molecule–110" (INCAM-110).
22. L. A. Liotta, *Am. J. Pathol.* **117**, 339 (1984); J. B. McCarthy, M. L. Basara, S. L. Palm, D. F. Sas, L. T. Furcht, *Cancer Metastasis Rev.* **4**, 125 (1985); G. L. Nicolson, T. Irimura, R. Gonzalez, E. Ruoslahti, *Exp. Cell Res.* **135**, 461 (1981); V. P. Terranova, J. E. Williams, L. A. Liotta, G. R. Martin, *Science* **226**, 982 (1984); M. J. Humphries, K. Olden, K. M. Yamada, *ibid.* **233**, 467 (1986); P. A. Netland and B. R. Zetter, *Biochem. Biophys. Res. Commun.* **139**, 515 (1986).
23. L. J. Old, *Science* **230**, 630 (1985); B. Beutler and A. Cerami, *Nature* **320**, 584 (1986).
24. R. S. Cotran and and J. S. Pober, *Kidney Int.* **35**, 969 (1989).
25. D. Spriggs, C. Imamura, C. Rodriguez, J. Horiguchi, D. W. Kufe, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6563 (1987).
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Vascular Endothelial Growth Factor Is a Secreted Angiogenic Mitogen

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Vascular endothelial growth factor (VEGF) was purified from media conditioned by bovine pituitary folliculostellate cells (FC). VEGF is a heparin-binding growth factor specific for vascular endothelial cells that is able to induce angiogenesis in vivo. Complementary DNA clones for bovine and human VEGF were isolated from cDNA libraries prepared from FC and HL60 leukemia cells, respectively. These cDNAs encode hydrophilic proteins with sequences related to those of the A and B chains of platelet-derived growth factor. DNA sequencing suggests the existence of several molecular species of VEGF. VEGFs are secreted proteins, in contrast to other endothelial cell mitogens such as acidic or basic fibroblast growth factors and platelet-derived endothelial cell growth factor. Human 293 cells transfected with an expression vector containing a bovine or human VEGF cDNA insert secrete an endothelial cell mitogen that behaves like native VEGF.

THE ELUCIDATION OF THE FACTORS that control angiogenesis is critical for the understanding of organ development and remodeling during embryonic and fetal life, wound healing, and tissue regeneration, as well as for insight into the pathogenesis of abnormal events such as neoplastic proliferations, rheumatoid arthritis, and retinopathies (*1*).

Several factors, including epidermal growth factor, transforming growth factors α and β, tumor necrosis factor, angiogenin, and prostaglandin E_2 are angiogenic in vivo (*2*). However, these agents have little or no direct mitogenic effect on vascular endothelial cells. Their action is thought to be mediated by other angiogenic inducers, either derived from macrophages (*1, 3*) or stored in the basement membrane (*4*). In contrast, basic and acidic fibroblast growth factors (bFGF and aFGF) are very effective in inducing vascular endothelial cell proliferation in vitro and angiogenesis in vivo (*5*). In view of their wide tissue distribution, FGFs have been proposed to be major mediators of angiogenesis (*5*). However, a puzzling aspect of the two FGFs casts doubts on their role as general mediators of angiogenesis: they both lack a hydrophobic signal peptide (*6*) required for the extracellular transport according to classical secretory pathways (*7*). Accordingly, FGFs are sequestered inside the cells of origin and apparently do not have direct access to target cells (*8*). FGF may be incorporated into the basement membrane and released when specific enzymes degrade this structure (*4*). Even the newly identified platelet-derived endothelial cell growth factor (PD-ECGF) lacks a signal peptide (*9*). There is, however, strong experimental evidence that angiogenesis requires the release of diffusible factors (*10*). Furthermore, media conditioned by a variety of transformed and untransformed cells exert mitogenic activity on endothelial cells (*11*), suggesting the secretion of mitogens distinct from FGF.

We identified and purified a heparin-binding vascular endothelial growth factor (VEGF) from media conditioned by bovine pituitary follicular or folliculostellate cells (FC) (*12*). This growth factor is a dimeric protein with a molecular mass of 45,000, composed of two subunits of identical molecular mass (23,000) and has a unique NH_2-terminal amino acid sequence. VEGF is a potent mitogen for vascular endothelial cells isolated from both small and large vessels, but does not affect the growth of BHK-21 fibroblasts, lens epithelial cells, corneal endothelial cells, keratinocytes, or adrenal cortex cells. The presence of VEGF in high concentrations in the medium conditioned by FC suggested that VEGF is a secreted molecule and that it may be a soluble mediator of endothelial cell growth and angiogenesis.

We isolated cDNA clones that encode bovine VEGF by screening a cDNA library (*13*) prepared from FC (*14*). This was done with a 59-base nucleotide probe (*15*) based on the NH_2-terminal amino acid sequence (Fig. 1A, position 2 to 21) of VEGF. Twenty hybridizing clones were identified in a library of 1.5×10^6 clones and two of these were sequenced for all the coding and for much of the noncoding regions. Their sequences were identical. The complete cDNA and corresponding protein sequence of one VEGF clone, bVEGF.6, is shown in Fig. 1A. This bovine VEGF cDNA clone contains an open reading frame of 190 amino acids. The NH_2-terminal amino acid sequence determined from the purified native bovine VEGF is preceded by 26 amino acids beginning with a methionine. The DNA sequence CCGAAACC preceding the ATG codon encoding the methionine agrees well with the initiation site consensus sequence (GCC)GCC$^{A}_{G}$CCATG in vertebrates (*16*). These 26 residues contain a hydrophobic core of 16 amino acids flanked by polar or charged residues indicative of a secretory signal sequence (*7*). The amino acid sequence Ser-Gln-Ala at positions −3 to −1

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