Journal of Cellular Biochemistry 78:674–680 (2000)

Hypoxia Induces Differential Expression of the Integrin Receptors $\alpha_{v\beta3}$ and $\alpha_{v\beta5}$ in Cultured Human Endothelial Cells

Harry L. Walton, Martha H. Corjay, Seema N. Mohamed, Shaker A. Mousa, Linda D. Santomenna, and Thomas M. Reilly*

Cardiovascular Diseases Research, DuPont Pharmaceuticals Company, Wilmington, Delaware 19880-0400

Abstract The integrins $\alpha_{v\beta3}$ and $\alpha_{v\beta5}$ have been implicated in playing a key role in the process of angiogenesis. In this study, we examined the effects of hypoxia, an important stimulus of angiogenesis, on the differential expression of the integrin subunits β_3 and β_5. β_3 and β_5 messenger RNA (mRNA), protein levels, and $\alpha_v\beta_3$ function were measured in human umbilical vein endothelial cells (HUVECs) cultured under normoxic and hypoxic (1% O_2) conditions. Cells exposed to hypoxic conditions for up to 72 h showed gradually increased mRNA levels of α_V and β_3, peaking at 24 h, in comparison with cells cultured under normoxic conditions. However, β_5 mRNA levels, under the same hypoxic conditions, remained at a constant level. Results from Western blot analysis of HUVECs, cultured under hypoxic conditions, paralleled those of the Northern analysis with an increased expression in $\alpha_v\beta_3$ protein levels, measured by blotting with LM609, evident by 24 h. $\alpha_v\beta_5$ protein levels, measured by blotting with P1F6, did not change for up to 72 h. HUVECs cultured under hypoxic conditions for 72 h showed increased attachment to fibrinogen, an $\alpha_v\beta_3$ mediated process. These results indicate that hypoxia can increase expression of $\alpha_v\beta_3$ in HUVECs, and that hypoxic regulation of $\alpha_v\beta_3$ may be an important regulator of angiogenesis. J. Cell. Biochem. 78:674–680, 2000. © 2000 Wiley-Liss, Inc.

Key words: hypoxia; $\alpha_{v\beta3}$; $\alpha_{v\beta5}$; vitronectin; antibody

INTRODUCTION

Angiogenesis is a process by which new blood vessels are formed from preexisting capillaries or postcapillary venules [Noden, 1989]. Angiogenesis is a critical process during development, wound healing [Folkman and Shing, 1992; Clark et al., 1982], and various diseases including cancer [Blood and Zetter, 1990], adult blindness [Aiello et al., 1995], and inflammatory disorders [Weinstat-Saslow and Steeg, 1994]. In adult organisms, vascular cells remain quiescent unless ischemia or hypoxic conditions stimulate the secretion of angiogenic factors [Patz, 1980; Shwerk et al., 1992]. Hypoxia, a state of reduced oxygen supply to tissue, is therefore an important regulator of blood vessel tone and structure [Arnet et al., 1996]. Hypoxia stimulates production of a number of angiogenic molecules in different cellular systems, including vascular endothelial growth factor (VEGF) in cultured human endothelial cells [Namiki et al., 1995] and in cultured smooth muscle cells [Stavri et al., 1995; Brogi et al., 1994], nitric oxide synthase and nitric oxide in endothelial cells [Arnet et al., 1996; Xu et al., 1995], and platelet-derived growth factor in endothelial cells [Kourembanas et al., 1991].

The integrin $\alpha_{v\beta3}$ is a major adhesion receptor [Cheresh and Spiro, 1987] for Arg-Gly-Asp-containing proteins including fibrinogen, vitronectin, von Willebrand factor [Cheresh, 1987], thrombospondin [Lawler et al., 1988], osteopontin [Reinholt et al., 1990], fibronectin [Charo et al., 1990], and laminin [Kramer et al., 1990]. This receptor is expressed on certain activated leukocytes, and on macrophages and osteoclasts [Ruoslahti and Pierschbacher, 1986; Davies et al., 1989; Horton et al., 1991]. It appears most prominently on cytokine-

*Correspondence to: Thomas M. Reilly, Cardiovascular Diseases Research, DuPont Pharmaceuticals Company, P. O. Box 80400, Wilmington, Delaware 19880-0400. E-mail: Thomas.M.Reilly@dupontpharma.com

Received 14 December 1999; Accepted 13 March 2000

Print compilation © 2000 Wiley-Liss, Inc.

activated endothelial and smooth muscle cells, and on vascular cells within human tumors and granulation tissue [Brooks et al., 1994b]. The highly restricted expression of $\alpha_{v\beta3}$ and its upregulation during angiogenesis suggest that it plays a critical role in angiogenic processes [Brooks et al., 1994a; Sepp et al., 1994]. A number of recent experimental studies with antibody or cyclic peptide antagonists of integrin $\alpha_{v\beta3}$ support this notion in that they are potent inhibitors of angiogenesis in different models, including the chick chorioallantoic membrane (CAM) [Brooks et al., 1994b], the murine retinopathy of prematurity (ROP) model [Luna et al., 1996], and murine tumor growth and metastases models [Varner and Cherish, 1996].

In contrast to $\alpha_{v\beta3}$, $\alpha_{v\beta5}$ is among the most widely expressed members of the integrin family and has been detected on most normal and transformed cells [Pasqualini et al., 1993]. Under certain conditions, $\alpha_{v\beta5}$ may also stimulate angiogenesis independent of $\alpha_{v\beta3}$ [Friedlander et al., 1995]. Because of the importance of hypoxia in stimulating pathological angiogenesis, in this study we investigated whether hypoxia can modulate $\alpha_{v\beta3}$ and $\alpha_{v\beta5}$ mRNA and protein levels in HUVECs.

MATERIALS AND METHODS

Reagents

Cells, culture media, and additives were from Clonetics (Walkersville, MD). [α-^{32}P]dCTP and *Redi*prime DNA labeling system were from Amersham Life Science, Inc.(Arlington Heights, IL). Other reagents were from standard suppliers or as listed in the text. The human integrin β_5 subunit was cloned using the published sequence [Ramaswamy and Hemler, 1990; McLean et al., 1990] by RT-PCR from total human placental RNA (CLONTECH Laboratories, Inc., Palo Alto, CA). First strand cDNA was prepared using oligo $dT_{12\text{-}18}$ and a gene specific primer. The β_5 cDNA was obtained in three segments by subjecting one-tenth of the RT mix to multiple rounds of PCR amplification using Deep Vent® DNA Polymerase (New England Biolabs, Inc., Beverly, MA). The individual segments were then assembled into a full-length cDNA using the cloning vector pNoTA/T7 (5 Prime ◠ 3 Prime, Boulder, CO) prior to subcloning into the eukaryotic expression vector, pcDNA3 (Invitrogen Corporation, Carlsbad, CA). The accuracy of the clone was confirmed by DNA sequence analysis, and its functionality was determined by in vitro transcription/translation using the TNT® Coupled Reticulocyte Lysate System (Promega, Madison, WI).

Cell Culture

HUVECs, pooled from three to five donors, were cultured in vented T162 flasks (Costar). Cells were maintained in endothelial cell growth medium with 2% fetal bovine serum. In these studies, cells from Lot P059 were used at passage three. Cells were maintained in Model 2700 (Cellstar) humidified incubators, with 5% CO_2, and either a balance of air (normoxic) or a balance of 1% O_2 in nitrogen (hypoxic). O_2 concentration was set using the control panel and calibrated by oxygen analyzer (Sensitron Associates, Reading, PA).

RNA Purification and Northern Blot Analysis

Following incubation under normoxic or hypoxic conditions for various times, cells were harvested and total RNA was isolated using an RNA Isolation Kit (Stratagene Cloning Systems, La Jolla, CA). Total RNA (10 μg/lane, in ethidium bromide) was denatured and electrophoresed on 1% agarose gels containing formaldehyde solution. After confirming integrity, RNA was transferred to a nylon membrane and immobilized by UV crosslinking, using a Stratalinker (Stratagene). The α_V (Oxford Biomedical Research, Inc., Oxford, MI), β_3 (Oxford), β_5, and β-actin (CLONTECH) cDNAs were labeled using a *redi*prime DNA labeling system and [α-^{32}P]dCTP, following methods provided by the vendor. The size of the α_V and β_3 plasmid inserts were 1.6 and 2.2 Kbs respectively [Fitzgerald et al., 1987]; β_5 was 2.4 Kb; the β-actin plasmid insert was 2.0 Kb [Findell et al., 1993]. The blots were probed with α-^{32}P-labeled α_V, β_3, or β_5 fragments, washed under standard conditions, and exposed to X-ray film (Kodak BioMax MS). To confirm equivalent loading of RNA per lane, membranes were then stripped of radioactivity and probed with α-^{32}P-labeled β-actin cDNA.

Western Analysis

HUVECs cultured under normoxic or hypoxic conditions were harvested, washed with ice-cold phosphate-buffered saline, divided into aliquots of 10^7 cells, pelleted by centrifugation,

and resuspended in biotinylation buffer. Cell surface proteins were labeled with biotin and immunoprecipitated using the Cellular Labeling and Immunoprecipitation Kit (Boehringer Mannheim Corporation, Indianapolis, IN). Immunoprecipitation was performed using LM609, a specific $\alpha_v\beta_3$ monoclonal antibody [Cheresh and Spiro, 1987; Chuntharapai et al., 1993], or P1F6, a specific $\alpha_v\beta_5$ monoclonal antibody [Friedlander et al., 1995]. In control experiments, a murine IgG monoclonal antibody that does not bind either $\alpha_v\beta_3$ or $\alpha_v\beta_5$ was used. Protein-antibody complexes were analyzed by electrophoresis on precast 10-18% SDS-polyacrylamide gels (Integrated Separation Systems) and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Sigma) using standard Western blot protocols. Biotin-labeled proteins were visualized using a BM Chemiluminescence Western Blotting Kit (Boehringer Mannheim) and exposure to X-ray film (Kodak BioMax MS).

Cell Attachment Assay

HUVECs grown under normoxic or hypoxic conditions were harvested, and 10^6 cells/mL were loaded with 2 μM calcein-AM. 100,000 calcein-loaded cells were plated onto wells of 96-well high-binding plates (Corning), which had been precoated with 25 μg/well purified human fibrinogen. The plates were incubated in the dark for one h at room temperature, washed to remove nonadherent cells, and then read on a Cytofluor II plate reader (Perceptive Biosystems) at excitation wavelength 485 nm and monitoring wavelength 530 nm.

RESULTS

Hypoxia Increases α_v and β_3 mRNA levels in HUVECs

Northern blot analysis of HUVEC mRNA after hybridization with cDNA probes for α_V and β_3 showed a marked increase in α_V and β_3 mRNA levels in cells exposed to hypoxic conditions (Fig. 1). The rise in the expression of these transcripts was evident after 12–18 h of reduced O_2 incubation, reached a maximum within 24 h, and persisted up to 72 h. No increase in β_5 mRNA levels was observed over the same time course. The α_V integrin mRNA was approximately 8.5 Kb, the β_3 integrin mRNA was approximately 6.6 Kb, and the β_5 integrin mRNA was approximately 3.3 Kb. Cells grown under normal O_2 conditions failed to show any increase in α_V, β_3, or β_5 expression (data not shown). The β-actin bands demonstrate equivalent RNA loading per lane. Each experiment was performed at least two times, and results from one representative experiment are shown in each case.

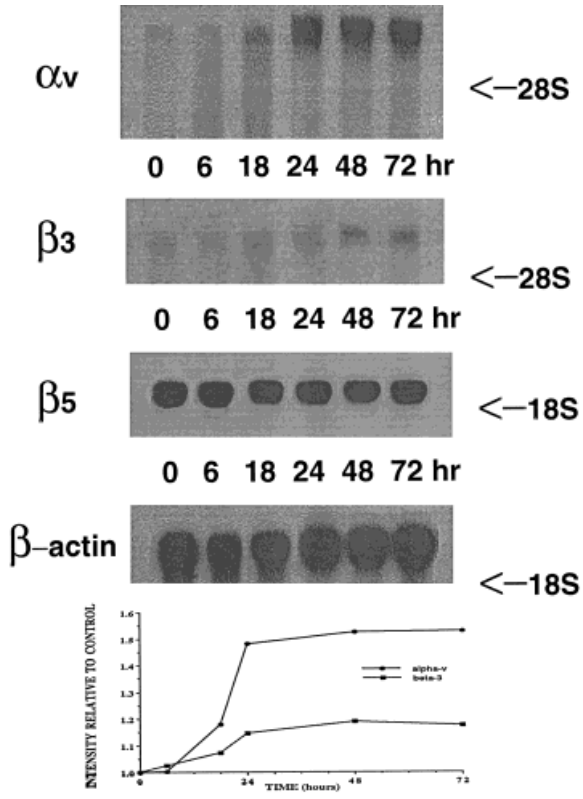

Fig. 1. The time course expression of α_v (top), β_3 (middle), β_5 (bottom), and β-actin (insert) mRNA in HUVECs exposed to hypoxia (1% O2) for 0, 6, 18, 24, 48, and 72 h was determined by Northern blot analysis of total RNA (10 μg per lane) extracted from confluent HUVEC cultures. Blots were first probed with either α-^{32}P-labeled α_v or α-^{32}P-labeled β_3 cDNA, then stripped and reprobed with α-^{32}P-labeled β-actin cDNA. Positions of the 28S and 18S bands are shown for reference. Relative intensity of α_v, β_3, and β_5 expression from densitometric scans of autoradiographs are shown in the graph.

Hypoxia Increases $\alpha_v\beta_3$ protein expression in HUVECs

Western blot analysis of HUVECs immunoprecipitated with LM609 showed a marked increase in $\alpha v\beta 3$ levels in cells exposed to hypoxic conditions (Fig. 2a). Under reduced conditions, αv appears as a major band at approximately 110 kDa and a minor band at approximately 40 kDa, which follows a previously reported pat-

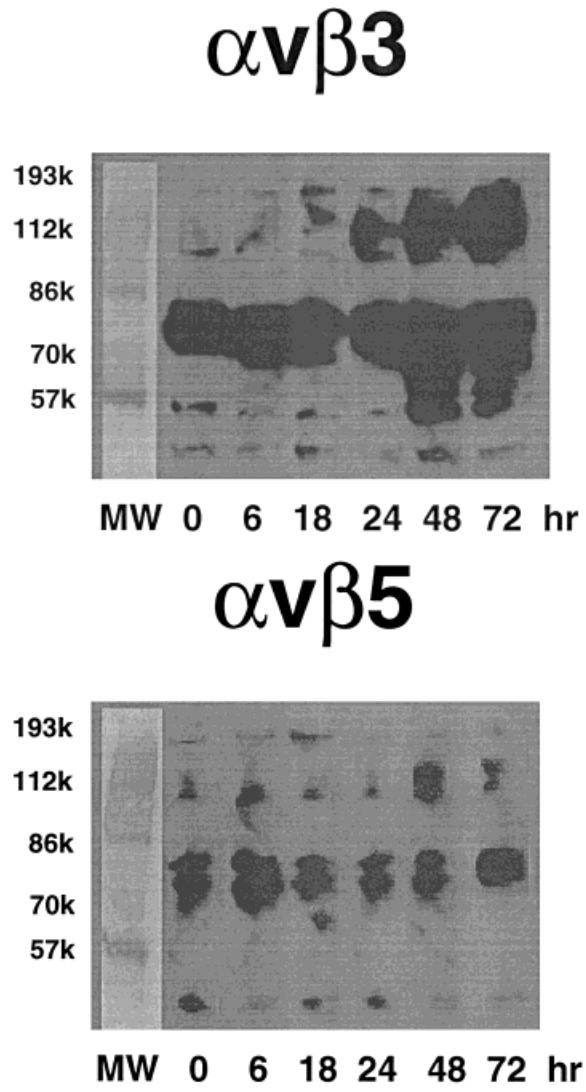

Fig. 2. **a**: Western analysis of immunoprecipitated HUVECs treated in hypoxic conditions for times indicated showing increasing $\alpha_{v\beta3}$ expression over 72 h**.** Molecular weight markers are shown for reference. The major α_v and β_3 bands are marked. **b:**Western analysis of immunoprecipitated HUVECs treated in hypoxic conditions for times indicated showing constant $\alpha_{v\beta5}$ expression over 72 h**.** Molecular weight markers are shown for reference. The major α_v and β_5 bands are marked.

tern [Gerber et al., 1996]. $_{\beta3}$ appears as a single band at approximately 85 kDa. As observed for mRNA levels, the rise in protein levels was by 24 h, and persisted up to 72 h. Western blot analysis of HUVECs immunoprecipitated with P1F6 showed no increase in $_{\alpha v\beta5}$ levels in cells exposed to hypoxic conditions during this same time period (Fig. 2b). As in Figure 2a, $_{\alpha v}$ appears as a major and a minor band. $_{\beta5}$ appears as a single band at approximately 85 kDa.

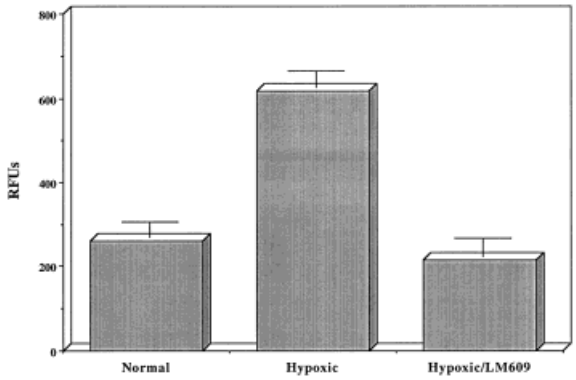

Fig. 3. Effect of hypoxia (1% O_2) on adhesion of HUVECs following 72 h treatment, compared to cells cultured under normoxic conditions for the same time (n=6). The ordinate shows signal intensity measured in relative fluorescent units (RFUs). Treatment of hypoxic cells by LM609 (0.1 μmol/L) reduced adhesion to normoxic levels (n=3). Similar treatment of normoxic cells by LM609 reduced adhesion to below detection threshold (data not shown).

Cells grown under normoxic conditions failed to show any increase in $_{\alpha v\beta3}$ or $_{\alpha v\beta5}$ protein (data not shown).

Effect of Hypoxia on HUVEC Adhesion

To assess the function of $\alpha_{v\beta3}$ protein produced under hypoxic conditions, adhesion assays to a fibrinogen matrix were performed. Under the conditions of this assay, HUVEC adhesion was an $\alpha_{v\beta3}$-dependent process [Mousa et al., 1996]. As shown in Figure 3, adhesion to fibrinogen was substantially increased in cells exposed to hypoxia, in comparison to those exposed to ambient atmosphere. In order to demonstrate that increased adhesion was due to increased $\alpha_{v\beta3}$ expression, hypoxic cells were treated with LM609, an antibody specific for $\alpha_{v\beta3}$ [Cheresh and Spiro, 1987; Chuntharapai et al., 1993], prior to the assay. As also shown in Figure 3, this pretreatment reduced adhesion to control levels, indicating that increased adhesion was due to $\alpha_{v\beta3}$. High background precluded accurate measurement of adhesion during time points earlier than 72 h.

DISCUSSION

To our knowledge this study represents the first report showing that hypoxia modulates $\alpha_{v\beta3}$ mRNA and protein levels, and the first report showing a differential response to hypoxia by $\alpha_{v\beta3}$ and $\alpha_{v\beta5}$. Our results demonstrate

that hypoxia leads to a parallel, time-dependent increase of α_v and $_{\beta 3}$ mRNAs and $\alpha_{v\beta 3}$ protein levels in HUVECs. The response of HUVECs to hypoxia therefore is not limited to increased levels of the $\alpha_{v\beta 3}$ mRNA, but includes production of functional $\alpha_{v\beta 3}$ protein as well. As assessed in adhesion assays to fibrinogen-coated wells, the $\alpha_{v\beta 3}$ induced by hypoxia in HUVECs was also manifested as a functional difference. In contrast, our results show no increase in either β_5 mRNA or $\alpha_{v\beta 5}$ protein levels in HUVECs under the same hypoxic conditions. Taken together, these results suggest that hypoxia may constitute a physiologic stimulus for $\alpha_{v\beta 3}$, but not $\alpha_{v\beta 5}$.

Previous studies have demonstrated the important role played by integrin $\alpha_{v\beta 3}$ in the process of angiogenesis [Brooks et al., 1994a; Brooks et al., 1994b; Luna et al., 1996; Friedlander et al., 1996]. $\alpha_{v\beta 3}$ is minimally expressed on resting or normal blood vessels, but is significantly upregulated on vascular cells within human tumors and granulation tissue, and in response to certain growth factors, such as basic fibroblast growth factor (bFGF) [Sepp et al., 1994] and TNFα, in vitro [Varner and Cheresh, 1996]. Antagonists of $\alpha_{v\beta 3}$ inhibit angiogenesis in a number of animal models [Brooks et al., 1994a; Varner and Cheresh, 1996; Friedlander et al., 1996], including the retinopathy of prematurity murine model. The mechanism of action of $\alpha_{v\beta 3}$ antagonists in blocking angiogenesis appears to be related to their ability to selectively promote apoptosis of newly sprouted blood vessels [Brooks et al., 1994b]. In view of the important role for integrin $\alpha_{v\beta 3}$ in angiogenesis, it is logical to expect that hypoxia, a fundamental stimulus for angiogenesis, would modulate expression levels of this integrin. It is interesting to note that levels of both the α_v and $_{\beta 3}$ subunits were increased by hypoxia. The α_v subunit is widely expressed on most cell types and associates with several different $_{\beta}$ subunits [Varner and Cheresh, 1996]. Integrin $\alpha_{v\beta 3}$ expression therefore is most likely regulated by $_{\beta 3}$ transcription.

Recent studies suggest that bFGF- and VEGF-induced angiogenesis may depend on distinct α_v integrins: $\alpha_{v\beta 3}$ and $\alpha_{v\beta 5}$ respectively [Friedlander et al., 1995; Varner, 1997]. Since angiogenesis is a critical biological process, it is not surprising that it may depend on redundant processes. $\alpha_{v\beta 5}$ may therefore serve as an alternate mechanism of angiogenesis in the absence of the β_3 subunit, as it does in individuals with Glansmann's thrombostenia [Friedlander et al., 1995]. The results of this study however, suggest that β_5, unlike β_3, does not respond to hypoxia.

Clearly, it will be important to elucidate the mechanism by which hypoxia influences integrin $\alpha_{v\beta 3}$ expression. The ability of VEGF to modulate $\alpha_{v\beta 3}$ expression on dermal endothelial cells suggests that hypoxia's effect in modulating $\alpha_{v\beta 3}$ levels may be mediated by increasing VEGF levels [Senger et al., 1996]. An analysis of the 3' region flanking the human VEGF gene revealed the presence of a sequence homologous to the hypoxia-responsive enhancer element of the human erythropoietin gene [Tischer et al., 1991; Nishida et al., 1993; Madan and Curtin, 1993; Blanchard et al., 1992]. No such sequence has been identified in the known published sequences of the α_v, β_3, or β_5 genes [Suzuki et al., 1987; Frachet et al., 1990; McLean et al., 1990], suggesting that the hypoxic effect on $\alpha_{v\beta 3}$ expression may be an indirect one. Further studies to address this hypothesis are in progress.

REFERENCES

Aiello LP, Pierce EA, Foley ED, Takagi H, Chen H, Riddle L, Ferra N, King GL, Smith LEH. 1995. Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. Proc Natl Acad Sci USA 92: 10457–10461.

Arnet UA, McMillan A, Dinerman JL, Ballermann B, Lowenstein CJ. 1996. Regulation of endothelial nitric-oxide synthase during hypoxia. J Biol Chem 271:15069–15073.

Blanchard KL, Acquaviva AM, Galson DL, Bunn HF. 1992. Hypoxic induction of the human erythropoietin gene: cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. Mol Cell Biol 12:5373–5385.

Blood DH, Zetter BR. 1990. Tumor interactions with the vasculature: angiogenesis and tumor metastasis. Biochim Biophys Acta 1032:89–118.

Brogi E, Wu T, Namiki A, Isner JM. 1994. Indirect angiogenic cytokines upregulate VEGF and bFGF gene expression in vascular smooth muscle cells, whereas hypoxia upregulates VEGF expression only. Circulation 90: 649–652.

Brooks PC, Clark RAF, Cheresh DA. 1994a. Requirement of vascular integrin $\alpha_{v\beta 3}$ for angiogenesis. Science 264: 569–571.

Brooks PC, Montgomery AMP, Rosenfeld M, Reisfeld RA, Hu T, Klier G, Cheresh DA. 1994b. Integrin $\alpha_{v\beta 3}$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell 79:1157–1164.

Charo IF, Nannizzi L, Smith JW, Cheresh DA. 1990. The vitronectin receptor $\alpha_{v\beta3}$ binds fibronectin and acts in concert with $\alpha_{5\beta1}$ in promoting cellular attachment and spreading on fibronectin. J Cell Biol 111:2795–2800.

Cheresh DA. 1987. Human endothelial cells synthesize and express an arg-gly-asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. Proc Natl Acad Sci USA 84:6471–6475.

Cheresh DA, Spiro RC. 1987. Biosynthetic and functional properties of an arg-gly-asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen, and von Willebrand factor. J Biol Chem 262: 17703–17711.

Chuntharapai A, Bodary S, Horton M, Kim K.1993. Blocking monoclonal antibodies to $\alpha_{v\beta3}$ integrin: a unique epitope of $\alpha_{v\beta3}$ integrin is present on human osteoclasts. Exp Cell Res 205:345–352.

Clark RAF, Della Pelle P, Manseau I, Lanigan JM, Dvorak HF, Colvin RB. 1982. Blood vessel fibronectin increases in conjunction with endothelial cell proliferation and capillary ingrowth during wound healing. J Invest Dermatol 79:269–276.

Davies J, Warwick J, Totty N, Philp R, Helfrich M, Horton M. 1989. The osteoclast functional antigen, implicated in the regulation of bone resorption, is biochemically related to the vitronectin receptor. J Cell Biol 109:1817–1826.

Findell PR, Wong KH, Jackman JK, Daniels DV. 1993. β_1-andrenergic and dopamine (D_1)-receptors coupled to adenylyl cyclanse activation in GT1 gonadotropin-releasing hormone neurosecretory cells. Endocrinology 312:682–688.

Fitzgerald LA, Pincz M, Steiner B, Rall SC, Bennett JS, Phillips DR. 1987. Comparison of cDNA-derived protein sequences of the human fibronectin and vitronectin receptor α-subunits and platelet glycoprotein IIb. Biochemistry 26:8158–8165.

Folkman J, Shing Y. 1992. Angiogenesis. J Biol Chem 267:10931–10934.

Frachet P, Uzan G, Thevenon D, Denarier E, Prandini MH, Marguerie G. 1990. GPIIb and GPIIIa amino acid sequences deduced from human megakaryocyte cDNAs. Mol Biol Rep 14:27–33.

Friedlander M, Brooks PC, Shaffer RW, Kincaid CM, Varner JA, Cheresh DA. 1995. Definition of two angiogenic pathways by distinct α_v integrins. Science 270:1500–1502.

Friedlander M, Theesfeld CL, Sugita M, Fruttiger M, Thomas MA, Chang S, Cheresh DA. 1996. Involvement of integrins $\alpha_{v\beta3}$ and $\alpha_{v\beta5}$ in ocular neovascular diseases. Proc Natl Acad Sci USA 93:9764–9769.

Gerber DJ, Periera P, Huang SY, Pelletier C, Tonegawa S. 1996. Expression of α_v and β_3 integrin chains on murine lymphocytes. Proc Natl Acad Sci USA 93:14698–14703.

Horton MA, Taylor ML, Arnett TR, Helfrich MH. 1991. Arg-Gly-Asp (RGD) peptides and the anti-vitronectin receptor antibody 23C6 inhibit dentine resorption and cell spreading by osteoclasts. Exp Cell Res 195:368–375.

Kourembanas S, Marsden PA, Mcquillan LP, Faller DV. 1991. Hypoxia induces endothelin gene expression and secretion in cultured human endothelium. J Clin Invest 88:1054–1057.

Kramer RH, Cheng Y-F, Clyman R. 1990. Human microvascular endothelial cells use β_1 and β_3 integrin receptor complexes to attach to laminin. J Cell Biol 111:1233–1243.

Lawler J, Weinstein R, Hynes RO. 1988. Cell attachment to thrombospondin: the role of RGD and integrin receptors. J Cell Biol 107:2351–2361.

Luna J, Tobe T, Mousa SA, Reilly TM, Campochiaro PA. 1996. Antagonists of integrin $\alpha_{v\beta3}$ inhibit retinal neovascularization in a murine model. Lab Invest 75:563–573.

Madan A, Curtin PT. 1993. A 24-base-pair sequence 3' to the human erythropoietin gene contains a hypoxia-responsive transcriptional enhancer. Proc Natl Acad Sci USA 90:3928–3932.

McLean JW, Vestal DJ, Cheresh DA, Bodary SC. 1990. cDNA sequence of the human integrin β_5 subunit. J Biol Chem 265:17126–17131.

Mousa SA, Forsythe M, Lorelli W, Bozarth J, Xue C-H, Wityak J, Sielecki TM, Olson RE, DeGrado W, Kapil R, Hussain M, Wexler R, Thoolen MJ, Reilly TM. 1996. Novel nonpeptide antiplatelet glycoprotein IIb/IIIa receptor antagonist, DMP754: receptor binding affinity and specificity. Coron Artery Dis 7:767–774.

Namiki A, Brogi E, Kearney M, Kim EA, Wu T, Couffinhal T, Varticovski L, Isner JM. 1995. Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells. J Biol Chem 270:31189–31195.

Nishida M, Carley WW, Gerritsen ME, Ellingsen O, Kelly RA, Smith TW. 1993. Isolation and characterization of human and rat cardiac microvascular endothelial cells. Am J Physiol 264: H639–H652.

Noden DM. 1989. Embryonic origins and assembly of blood vessels. Am Rev Respir Dis 140: 1097–1103.

Pasqualini RJ, Bodorova S, Hemler MF. 1993. A study of the structure, function and distribution of β_5 integrins using novel anti-β_5 monoclonal antibodies. J Cell Sci 105:101–111.

Patz A. 1980. Studies on retinal neovascularization. Invest Ophthalmol Vis Sci 19:1133–1138.

Ramaswamy H, Hemler ME. 1990. Cloning, primary structure and properties of a novel human integrin β subunit. EMBO J 9:1561–1568.

Reinholt FP, Hultenby K, Oldenberg A, Heinegard D. 1990. Osteopontin: a possible anchor of osteoclasts to bone. Proc Natl Acad Sci USA 87:4473–4475.

Ruoslahti E, Pierschbacher M. 1986. Arg-Gly-Asp: a versatile cell recognition sequence. Cell 44: 517–518.

Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Peruzzi CA, Detmar M. 1996. Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the $\alpha v\beta3$ integrin, osteopontin, and thrombin. Am J Pathol 149:293–305.

Sepp NT, Li L-J, Lee KH, Brown EJ, Caughman SW, Lawley TJ, Swerlick RA. 1994. Basic fibroblast growth factor increases expression of the $\alpha_{v\beta3}$ complex on human microvessel endothelial cells. J Invest Dermatol 103: 295–299.

Shwerk D, Itin A, Soffer D, Keshtet E. 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-induced angiogenesis. Nature 359:843–845.

Stavri GT, Zachary IC, Baskerville PA, Martin JF, Erusalimsky JD. 1995. Basic fibroblast growth factor upregulates the expression of vascular endothelial growth factor in vascular smooth muscle cells. Circulation 92:11–14.

Suzuki S, Argraves WS, Arai H, Languino LR, Pierschbacher MD, Ruoslahti E.1987. Amino acid sequence of the vitronectin receptor alpha subunit and comparative expression of adhesion receptor mRNAs. J Biol Chem 262:14080–14085.

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA. 1991. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 266:11947–11954.

Varner JA. 1997. The role of vascular cell integrins $\alpha_{v\beta3}$ and $\alpha_{v\beta5}$ in angiogenesis. EXS 79:361–390.

Varner JA, Cherish DA. 1996. Tumor angiogensis and the role of vascular cell integrin $\alpha_{v\beta3}$. In: DeVita VT, Hellman S, Rosenberg SA, editors. Important Advances in Oncology. Philadelphia: Lippincott-Raven. p 69–87.

Weinstat-Saslow D, Steeg PS. 1994. Angiogenesis and colonization in the tumor metastatic process: basic and applied advances. FASEB J 8:401–407.

Xu X-P, Pollock JS, Tanner MA, Myers PR. 1995. Hypoxia activates nitric oxide synthase and stimulates nitric oxide production in porcine coronary resistance arteriolar endothelial cells. Cardiovasc Res 30:841–847.