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Induction of the Angiogenic Phenotype by Hox D3

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Abstract. Angiogenesis is characterized by distinct phenotypic changes in vascular endothelial cells (EC). Evidence is provided that the Hox D3 homeobox gene mediates conversion of endothelium from the resting to the angiogenic/invasive state. Stimulation of EC with basic fibroblast growth factor (bFGF) resulted in increased expression of Hox D3, integrin αvβ3, and the urokinase plasminogen activator (uPA). Hox D3 antisense blocked the ability of bFGF to induce uPA and integrin αvβ3 expression, yet had no effect on EC cell proliferation or bFGF-mediated cyclin D1 expression. Expression of Hox D3, in the absence of bFGF, resulted in enhanced expression of integrin αvβ3 and uPA. In fact, sustained expression of Hox D3 in vivo on the chick chorioallantoic membrane retained EC in this invasive state and prevented vessel maturation leading to vascular malformations and endotheliomas. Therefore, Hox D3 regulates EC gene expression associated with the invasive stage of angiogenesis.

Angiogenesis requires coordinate changes in endothelial cell morphology and gene expression. In the resting vasculature, quiescent capillary endothelial cells are in contact with a laminin-rich basement membrane (BM) and are nonmigratory. In response to angiogenic stimuli, including cytokines such as basic fibroblast growth factor (bFGF), tumor necrosis factor α, and vascular endothelial growth factor, vascular endothelial cells (EC) reenter the cell cycle and upregulate proteolytic activity to degrade the existing BM, facilitating their invasion into stromal tissue. These vascular sprouts then synthesize a new BM and undergo morphological reorganization into mature quiescent, lumen-containing capillaries.

Proteolysis of the BM, along with enhanced vascular permeability which accompanies angiogenesis, facilitates an influx of serum proteins including vitronectin, fibronectin, and fibrinogen, creating a provisional extracellular matrix on which EC migrate. Although not typically expressed by quiescent resting EC, angiogenic EC express high levels of integrin αvβ3, which can effectively bind this provisional matrix (Brooks et al., 1994; for review see Cheresh and Mecham, 1994). In fact, both matrix-degrading serine and metalloproteinases and integrin αvβ3 have been shown to play essential roles in new vessel formation, as inhibition of their respective activities will impair tumor- or cytokine-mediated angiogenesis (Mignatti et al., 1989; Brooks et al., 1994; Johnson et al., 1994; Min et al., 1996). The mechanism by which angiogenic cytokines coordinately upregulate expression of proteases and adhesion molecules involved in angiogenesis however is not known.

One class of transcriptional activators that have been linked to extensive tissue remodeling are the homeobox (Hox) genes. In addition to their role in embryonic development, the Hox genes have been shown to play a significant role in differentiation and gene expression in adult tissues (Lawrence and Largman, 1992; Takeshita et al., 1993; Savageau et al., 1995). Inappropriate Hox gene expression has also been linked to tumorigenic tissues (Friedmann et al., 1994; Redline et al., 1994). Hox genes are characterized by a highly conserved 180-bp DNA-binding domain known as the homeodomain, which interacts directly with DNA to activate transcription of genes (Desplan et al., 1985). Putative target genes for Hox DNA-binding proteins include other Hox genes and transcription factors, cell adhesion molecules, extracellular proteins, and growth factors (for reviews see Botas, 1993; and Edelman and Jones, 1993; Immergluck et al., 1990; Goober et al., 1994; Taniguchi et al., 1995). Interestingly, in the genome, Hox and integrin receptor gene families colocalize in clusters, indicative of parallel, coordinate evolution, further supporting a link between these groups of genes associated with tissue patterning (Wang et al., 1995).

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1. Abbreviations used in this paper: bFGF, basic fibroblast growth factor; BM, basement membrane; CAM, chorioallantoic membrane; EC, vascular endothelial cells; HUVEC, human umbilical vein endothelial cells; uPA, urokinase plasminogen activator.

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Given the dramatic changes in cell–extracellular matrix interactions, EC morphology, and gene expression that occur during angiogenesis, we investigated expression of Hox genes and their role in endothelial cell behavior.

Materials and Methods

Cells and Culture Conditions

Experiments were conducted using primary human umbilical vein endothelial cells (HUVECs; Clonetics, San Diego, CA) or an immortalized HUVEC cell line (line 199; American Type Culture Collection, Rockville, MD). HUVECs were routinely cultured in M199 plus 20% FCS and ECGS (Upstate Biotechnologies, Lake Placid, NY). The immortalized 1998 cell line was maintained in M199 plus 5% FCS. For experiments in which bFGF was added, HUVECs were maintained in M199 plus 5% FCS, while the 1998 cell line was maintained in M199 with 2% serum. Recombinant human bFGF was purchased from Upstate Biotechnologies, and also kindly supplied by Dr. Judith Abraham (Scios Nova Inc., Mountain View, CA). For experiments using basement membrane, 1 x 10^6 cells were seeded onto thick layers of Matrigel (Becton Dickinson, Bedford, MA). Chick embryo fibroblast isolation and maintenance of the viral packaging cell line Q4dh was performed as previously described (Stoker and Bissell, 1988).

Transfection of immortalized HUVEC or Q4dh cell lines was performed using calcium phosphate and stable transfectants selected using 800 or 400 µg/ml G418, respectively (Sigma Chemical Co., St. Louis, MO). At least two independently selected pools of stably transfected cells were used for subsequent analysis.

Isolation of HOX Genes Expressed in EC

RT-PCR of Hox genes was performed using total RNA from primary HUVECs cultured in the presence or absence of BM. After reverse transcription with oligo-dT primers (Gene Amp; Perkin Elmer, Norwalk, CT), DNA was amplified using degenerate primers against the conserved homeodomain sequences as described by Friedman et al. (1994); forward primer 5’-GGAATTCGCARGTNGARAARGARTT-3’ and reverse primer 5’-CCCAAGCTTTTGGAAACADATTT-3’. PCR reaction conditions include initial denaturation for 2 min at 95°C followed by 35 cycles at 95, 55, and 72°C for 1 min each plus a final incubation for 10 min at 72°C. In some instances, samples were reamplified for an additional 25 cycles using similar reaction conditions. Final PCR products were cloned into TA cloning vectors (Invitrogen, San Diego, CA) and positive clones amplified and HOX sequences identified by the dideoxy sequencing method of Sanger et al. (1977).

For quantitative PCR analysis of Hox D3 mRNA, 1 µg of total RNA was reverse transcribed using oligo-dT primers and amplified for 30 cycles at 95, 60, and 72°C with Hox D3 primers spanning a coding region to eliminate amplification of possible contaminating genomic DNA (forward primer [bp 1682-1705] 5’-TGGTCTGAACTCAGAGCAGCAGC-3’; and reverse primer [bp 3962-3938] 5’-TCATGCGCCGGTTCTGGAACCA-3’). This yielded the predicted 415-bp PCR product, which was confirmed by DNA sequencing. To normalize for amounts of starting RNA, equal volumes of reaction mixtures were amplified for 35 cycles at 95, 60, and 72°C with Hox D3 primers spanning a coding region to eliminate amplification of possible contaminating genomic DNA (forward primer [bp 1682-1705] 5’-TGGTCTGAACTCAGAGCAGCAGC-3’; and reverse primer [bp 3962-3938] 5’-TCATGCGCCGGTTCTGGAACCA-3’). This yielded the predicted 415-bp PCR product, which was confirmed by DNA sequencing. To normalize for amounts of starting RNA, equal volumes of reaction mixtures were amplified for 35 cycles at 95, 60, and 72°C with Hox D3 primers spanning a coding region to eliminate amplification of possible contaminating genomic DNA (forward primer [bp 1682-1705] 5’-TGGTCTGAACTCAGAGCAGCAGC-3’; and reverse primer [bp 3962-3938] 5’-TCATGCGCCGGTTCTGGAACCA-3’). This yielded the predicted 415-bp PCR product, which was confirmed by DNA sequencing. To normalize for amounts of starting RNA, equal volumes of reaction mixtures were amplified for 35 cycles at 95, 60, and 72°C with Hox D3 primers spanning a coding region to eliminate amplification of possible contaminating genomic DNA (forward primer [bp 1682-1705] 5’-TGGTCTGAACTCAGAGCAGCAGC-3’; and reverse primer [bp 3962-3938] 5’-TCATGCGCCGGTTCTGGAACCA-3’).

Construction of Expression Vectors

Human genomic Hox D3 DNA was a gift from Y. Taniguchi (Tokai School of Medicine, Boheisaidai, Isehara, Kanagawau, Japan). A full length Hox D3 cDNA was constructed by inserting the 415-bp PCR product, generated as described above, between the Pst I site (bp 1899) and the EcoRI site (bp 3853) in the genomic Hox D3. Expression vectors were constructed by inserting either the genomic or Hox D3 cDNA between the KpnI and BamHI sites of pcDNA3 mammalian expression vector (Invitrogen). Antisense expression vectors were constructed by inserting the genomic DNA between the BamHI and HindIII sites of the pcDNA3 in the antisense orientation. For controls, cells were transfected with empty pcDNA3 vectors.

Chicken retroviral expression vectors were constructed by inserting either the Hox D3 genomic DNA or cDNA between the ClaI and BamHI sites of the replication-defective retroviral vector CK (a gift from B. Venstrom, European Molecular Biology Laboratory, Heidelberg, Germany). These vectors were then stably transfected into the packing cell line Q4dh to generate infectious virus (Stoker and Bissell, 1988). Production of infectious retrovirus was monitored by infecting chick embryo fibroblasts with supernatants from cultures of stably transfected packing cells as described by Stoker and Bissell (1988).

RNA Isolation and Northern Blot Analysis

Total RNA was extracted using the Quick-Prep Kit (Qiagen, Santa Clara, CA). For cells cultured on BM, cells were first detached from Matrigel using Matrisperse (Becton Dickinson) and RNA isolated as described above. 10 or 20 µg total RNA was run through 1% agarose formaldehyde gels and transferred to Hybond-N membranes (Amersham Intl., Arlington Heights, IL) according to standard procedures. Membranes were hybridized using [32P]dCTP-labeled cDNA probes and exposed to Kodak X-Omat film at −70°C. cDNA probes for human β3 and β5 integrin were from E. Filardo (Brown University, Providence, RI). cDNA probe for human urokinase plasminogen activator (uPA) was kindly provided by Graham Parry (The Scripps Research Institute, La Jolla, CA).

Western Blots

Cultured cells were lysed in buffer containing 1 M NaCl, 10 mM Tris, pH 7.5, 1% Triton X-100 and aprotinin, PMSF, and leupeptin. Total protein concentration was determined using the BCA reagent kit (Pierce, Rockford, IL), and a total of 10 µg was run on 10 or 12% SDS-PAGE under reducing conditions. Gels were transferred to Immobilon nylon membranes (Millipore, Bedford, MA) and blocked in TBS plus 5% milk proteins. Blots were probed using either 1:200 dilution of monoclonal anti-human cyclin D1 (sc-246; Santa Cruz Biotechnologies, Santa Cruz, CA) or 10 µg/ml polyclonal rabbit anti-human β3 integrin (AP-3) followed by a 1:1,000 dilution of either goat anti–rabbit or goat anti–mouse HRP (Biosource International, Camarillo, CA) and visualized by chemiluminescence (ECL; Amersham Intl.).

Adhesion Assays

Cells were removed by incubation with versene (0.5 M EDTA in PBS, pH 7.4) and resuspended in FBMA media (Clonetics Corp., San Diego, CA) supplemented with 0.2 M MnCl. A total of 5 x 10^6 cells was seeded into each well of 24-well culture dishes (Costar Corp., Cambridge, MA) that had been previously coated for 1 h at 37°C with either heat-denatured BSA (1%) or 10 µg/ml human fibrinogen (Sigma Chemical Co.). In some cases 25 µg/ml of a function blocking anti-human αvβ3 integrin (mAb LM609) were added along with cells. After extensive washing, cells were fixed in 2% ethanol and stained with 1% crystal violet. The number of adherent cells was quantitated by absorbance at 600 nm.

BrDU Incorporation

To determine the effect of Hox D3 on cell proliferation, the rate of DNA synthesis was established by measuring BrDU incorporation in control and EC transfected with either Hox D3 or Hox D3 antisense expression vectors. After incubation for 4 or 12 h with 10 µM BrDU, cells were fixed with 70% ethanol and stained with an anti-BrDU kit (Boehringer Mannheim, Indianapolis, IN) followed by staining with 0.5 µg/ml DAPI (4,6 diamidino-2-phenylindole; Sigma Chemical Co.). The percentage of BrDU-positive nuclei was determined by counting at least six different fields in each of the cell types tested.

Chick Chorioallantoic Membrane Assay

All assays were performed in 10-d-old pathogen-free embryos (SPAFAS). Briefly, the chorioallantoic membranes (CAMs) were prepared as previously described by Brooks et al. (1994). 48 hr post infection with recombinant retrovirus, CAMs were harvested by washing in PBS and fixed for 30 min in 2% paraformaldehyde followed by washing in 0.1 M glycine/PBS. CAMs were embedded into embedding medium (OCT; Miles, Elkhart, IN), flash frozen in liquid nitrogen, and stored at −80°C. 5-µm sections were prepared and mounted on slides for immunohistochemistry.
Immunohistochemistry

Sections were briefly fixed in 100% acetone and equilibrated with PBS. After blocking in PBS plus 5% BSA, sections were incubated with either 5 μg/ml monoclonal antihuman αβ3 (LM609) or a 1:1,000 dilution of polyclonal rabbit anti-human von Willebrand Factor (BioGenex, San Ramon, CA). Goat anti-mouse-rhodamine or goat anti-rabbit-FITC (Biosource International, Camarillo, CA) were used as secondary antibodies. Sections were subsequently mounted using Fluoromount (Southern Biotechnologies, Birmingham, AL).

In Situ Hybridization

Hox D3 sense and antisense digoxigenin-labeled RNA probes were generated by linearizing the vector pCR II (Invitrogen) containing the 415-bp Hox D3 cDNA (generated by RT-PCR described above) and incubating with T7 or SP6 RNA polymerase and digoxigenin-conjugated dUTP (Genius; Boehringer Mannheim). In situ hybridization was performed on 3-μm cryosections mounted on siliconized slides (Sigma Chemical Co.). Sections were fixed in 4% paraformaldehyde for 20 min and dehydrated in 30, 70, and 100% ethanol for 2 min each and hybridized overnight at 45°C with 800 ng/ml of Hox D3 riboprobes in 50% formalamide, 10% dextran sulfate, 1% blocking reagent (DIG nucleic acid detection kit; Boehringer Mannheim), 300 μg/ml yeast tRNA (Sigma Chemical Co.), 3 mM NaCl, 10 mM Tris, pH 7.5, and 1 mM EDTA. After washing in 0.2× SSPE at 50°C for 2 h, sections were incubated overnight with a 1:500 dilution of HRP-conjugated antibodies against digoxigenin and color developed using NBT/BCIP (DIG nucleic acid detection Kit; Boehringer Mannheim). Nonspecific hybridization was assessed using Hox D3 sense riboprobes.

Results

Hox Gene Expression and the Angiogenic Phenotype of EC

EC cultured on basement membrane adopt a distinct morphology reminiscent of capillaries in vivo (Fig. 1 A, + BM), withdraw from the cell cycle, and cease DNA synthesis within 24 h (Kubota et al., 1988). This “differentiated phenotype” contrasts the characteristic cobblestone morphology associated with proliferating EC cultured in the absence of BM (Fig. 1 A, −BM). RT-PCR of mRNA from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown). Previous studies have linked Hox D3 from cultured EC detected the presence of a number of Hox gene mRNA’s including Hox A4, B3, B4, B5, and D3 (data not shown).

Hox D3 Induces β3 Integrin and uPA mRNA but Does Not Influence EC Proliferation

To determine whether the expression of Hox D3 influences expression of genes associated with the angiogenic phenotype of EC, a HUVEC cell line was stably transfected with a Hox D3 expression vector. Northern blot analysis shows that HUVECs overexpressing Hox D3 have significantly higher levels of steady-state β3 integrin and uPA mRNA levels as compared to cells transfected with control vectors. In contrast, mRNA encoding integrin β5, which is not upregulated during angiogenesis, did not differ in control or Hox D3-transfected cells (Fig. 2 A). To establish whether Hox D3 was required for EC expression of β3 integrin and uPA, HUVECs were stably transfected with Hox D3 antisense expression vectors. Compared to control-transfected cells, baseline levels of both β3 integrin and uPA mRNA were significantly reduced in cells expressing Hox D3 antisense. In contrast, levels of β5 integrin mRNA were not influenced by expression of Hox D3 antisense (Fig. 2 B).

The Hox D3-mediated changes in expression of integrin β3 mRNA also resulted in corresponding changes in surface expression of functional αβ3 integrin. We compared the ability of EC overexpressing Hox D3 or antisense against Hox D3 to adhere to fibrinogen, a ligand for αβ3 on endothelial cells (Cheresh et al., 1989). EC expressing antisense against Hox D3 displayed significantly reduced...
capacity to adhere to fibrinogen, compared to control cells (Fig. 3 A), whereas overexpressing Hox D3 increased EC adhesion to fibrinogen (Fig. 3 A). Treatment of cells with LM609, a function-blocking antibody against αvβ3 (Cheresh, 1987), resulted in a complete inhibition of the fibrinogen-mediated attachment of these cells (data not shown). Thus, Hox D3 regulates the functional expression of αvβ3 on endothelial cells.

The fact that angiogenesis is often accompanied by EC proliferation prompted us to examine whether Hox D3 also influenced the rate of EC growth. We therefore compared DNA synthesis, as determined by BrdU incorporation, in control EC and EC transfected with Hox D3 sense or antisense expression vectors. In contrast to the Hox D3-induced changes in expression of integrin β3 and uPA described above, no differences in DNA synthesis were observed between control EC, EC expressing Hox D3, or antisense against Hox D3 (Fig. 3 B). Therefore, while Hox D3 antisense can influence the expression of integrin αvβ3 and uPA in EC it apparently does not directly influence the proliferation of these cells.

**Hox D3 Mediates bFGF-induced Expression of β3 Integrin and uPA but Not Cyclin D1 in EC**

The angiogenic cytokine bFGF leads to expression of both uPA and β3 integrin in EC, which subsequently contribute to the invasive properties of EC during the early stages of angiogenesis (Moscatechi et al., 1985; Enenstein et al., 1992; Brooks et al., 1994; Sepp et al., 1994). To determine whether Hox D3 could influence the bFGF-mediated induction of integrin αvβ3 and uPA, EC were made quiescent by culturing on BM and treated with or without bFGF and analyzed for expression of mRNA encoding these proteins. As shown in Fig. 4, exposure of HUVECs to bFGF led to an increase in Hox D3 expression after 8 h, which was accompanied by increased β3 integrin and uPA steady-state mRNA levels by 24 h (Fig. 4, A and B). In contrast, in EC expressing antisense against Hox D3, the ability of bFGF to induce high levels of expression of β3 integrin and uPA mRNA expression was attenuated. Importantly, integrin β5 mRNA was not altered in either the control or antisense-transfected cells in the presence or absence of bFGF (Fig. 4, A and B).
Although bFGF can also act as an endothelial cell mitogen, expressing Hox D3 antisense had no effect on bFGF’s ability to induce cyclin D1 in EC (Fig. 4 C). Thus Hox D3 appears to mediate bFGF-induced expression of β3 integrin and uPA but does not directly influence endothelial cell cycle progression. This is consistent with the observation that Hox D3 antisense had no effect on EC proliferation (Fig. 3 B). These findings indicate that Hox D3 appears to influence EC genes important for invasion but not proliferation.

**Sustained Expression of Hox D3**

**In Vivo Results in Vascular Malformations and Development of Endotheliomas**

During the late stages of angiogenesis, integrin αvβ3 and uPA are down regulated as EC reestablish an intact BM and form a lumen, suggesting that Hox D3 may be required only during the initial stages of angiogenesis. Maintaining expression of Hox D3 therefore might be expected to prolong expression of an invasive phenotype and interfere with normal vascular maturation and/or remodelling. To test this possibility, we constructed replication-defective avian retroviral vectors designed to constitutively express human Hox D3 in vivo. Transfection of a transformed viral packaging cell line Q4dh with Hox D3 proviral vectors yielded a retrovirus capable of infecting proliferating embryonic chick cells and driving expression of human Hox D3 in these cells. When grafted onto 10-d chick embryo CAM’s, transformed virus-producing Q4dh cells generate solid tumors in these animals. The continued production of infectious virus leads to infection of adjacent, proliferating cells including tumor-associated EC. As shown in Fig. 5 A, maintenance of Hox D3 expression in these tissues caused blood vessel malformations, reminiscent of endotheliomas, which appeared as abnormal capillary-like structures several times greater in diameter than large capillaries and were filled with hematopoietic cells. A majority of the EC and certain hematopoietic cells within these endotheliomas remained positive for αvβ3, providing evidence that Hox D3 was active in these tissues (Fig. 5 B). To confirm the presence of Hox D3 in these vascular structures, we performed in situ hybridization using probes that detect retrovirally produced human Hox D3 but do not detect endogenous chick Hox genes. Retroviral-mediated Hox D3 expression was observed in a wide variety of tissues including epithelium, connective tissue, hematopoietic cells, and endothelial cells (Fig. 5 C). In contrast to the widespread viral infection and expression of Hox D3, αvβ3 integrin was only detected in EC and some hematopoietic cells, suggesting this expression was highly specific.

These vascular malformations or endotheliomas ultimately resulted in the formation of large hemorrhagic zones within the tumors generated by 15/18 Hox D3-infected embryos, but was not observed in tumors of the control embryos (Fig. 6, A and B). H&E staining of sectioned Hox D3-infected tissue showed large endothelial-lined cysts filled with hematopoietic cells, many of which had hemorrhaged (Fig. 6 D). In contrast, tumors producing control virus showed normal tumor-induced angiogenesis (Fig. 6 C). These findings not only establish a role for Hox D3 in EC function but also emphasize the requirement for tightly regulated expression of Hox D3 during the early events of angiogenesis.

**Discussion**

During embryonic development, normal tissue patterning depends upon temporally and spatially restricted expression of Hox genes. Evidence is presented in this report that patterning of the vasculature during angiogenesis is affected by expression of the Hox D3 homeobox gene in EC. We show that quiescent EC in contact with a BM express minimal levels of Hox D3, integrin αvβ3, and uPA. However, exposure of EC to the angiogenic cytokine bFGF leads to increased levels of Hox D3, resulting in the functional expression of the angiogenic-promoting genes integrin β3 and uPA in these cells.

Our findings that prolonged expression of Hox D3 results in abnormal vascular morphology also emphasizes
the requirement for tightly regulated expression of Hox genes in EC. Previous studies have linked sustained expression of angiogenic mediators, including bFGF and uPA with vascular tumors (Takahashi et al., 1994; Hatva et al., 1996; Kraling et al., 1996). In vivo, transgenic mice expressing polyoma middle T oncogene develop endothelial tumors, and in culture, these transformed EC form cystic vascular structures reminiscent of endothelioma, a phenotype that can be reversed by inhibiting uPA activity (Bautch et al., 1987; Montesano et al., 1990). In addition, embryonic chick cells infected with v-src, a downstream effector of FGF, show elevated uPA activity, and v-src infection of chick endothelial cells in vivo results in endotheliomas (Sullivan and Quigley, 1986; Stoker et al., 1990). Thus, while increased uPA activity is involved in normal capillary remodeling, chronic expression driven by Hox D3 would be expected to prevent EC from establishing basement membranes, forming patent vessels with lumens and resuming a quiescent, differentiated phenotype. Furthermore, the sustained expression of integrin αvβ3 provides the necessary means for EC to adhere, migrate, and survive in this angiogenic environment and thereby may enhance the activity of matrix-degrading serine and metalloproteinases, which interact with this integrin (Brooks et al., 1996; Strömblad et al., 1996).

Previous studies have linked FGF to the induction of Hox D genes during embryonic limb development, although the precise targets of Hox D gene activity have not been identified (Duprez et al., 1996). In this report it is apparent that Hox D3 induction of integrin αvβ3 is tissue specific, since this integrin was not detected in epithelial tissues expressing human Hox D3. In fact, endogenous

**Figure 5.** Colocalization of Hox D3 and αvβ3 integrin in endotheliomas in vivo. (A) H&E-stained cross-section of tissue infected by Hox D3-expressing retrovirus shows an abnormally large capillary-like structure filled with erythrocytes. (B) Immunofluorescent staining with LM609 against αvβ3 in the corresponding serial section shows strong positive staining in both the endothelial component (ec) of this vascular structure and in hematopoietic cells (h) contained within. (C) In situ hybridization of retrovirally expressed Hox D3 in corresponding serial section showing widespread expression in epithelium (ep), endothelial cells (ec), connective tissue (c), and hematopoietic cells (h). (D) Control in situ hybridization in a serial section performed with a sense riboprobe for Hox D3. Bar, 20 μm.

**Figure 6.** Effect of retroviral-mediated expression of Hox D3 in chick embryos. (A and B) Morphology of tumors generated 3 d after grafting of QT6 cells producing control or Hox D3-expressing retrovirus onto 10 d chick CAMs. (C and D) H&E staining in tissue cross-sections from tumors (t) and vasculature produced by cells shedding control or Hox D3-expressing retrovirus. Note the large hemorrhagic region containing hematopoietic cells (h) adjacent to tumor tissue in the Hox D3-infected tissue. Bar, 50 μm.
Hox D3 is expressed in human skin epithelium, yet this tissue does not express ovβ3 (Detmer et al., 1993; Brooks et al., 1994; Brown et al., 1994; Gailit et al., 1994). We also observed that quiescent vessels in the skin do not express Hox D3 (Boudreau, N., unpublished observations). However, EC and certain hematopoietic cells infected with Hox D3-expressing retrovirus subsequently stained positively for ovβ3. Hox D3 has previously been shown to induce expression of the β subunit of the αIIβ3 platelet adhesion receptor in cultured human erythroleukemia cells which can be induced to differentiate to erythroid cells (Taniguchi et al., 1995). Thus, the ability of Hox D3 to mediate expression of the β3 integrin subunit may be restricted to cells of the hematopoietic/angioblast lineage which arise from common blood islands during embryogenesis (Risau, 1995).

We have also shown that Hox D3 antisense blocks the bFGF-induced expression of integrin β3 and uPA mRNA, yet has no effect on bFGF-induced expression of cyclin D1 in these cells. Furthermore, we have shown that Hox D3 does not directly influence the rate of cell proliferation, and together these results suggest that these aspects of angiogenesis are differentially regulated. These observations are also supported by studies showing Hox D3 overexpression had no effect on cell proliferation (Taniguchi et al., 1995). Therefore in EC, Hox D3 appears to selectively regulate the expression of genes that contribute to extra-cellular matrix remodeling during angiogenesis.

Although both the integrin β3 and uPA promoters contain several potential Hox-binding consensus sequences, the complex relationship between Hox genes makes it difficult to predict whether Hox D3 acts alone or in combination with sequentially activated Hox genes to induce expression of these angiogenic effectors. For example, studies with compound mutants of Hox D3 and A3, suggest that paralogous Hox genes act in a synergistic manner to influence tissue patterning (Condie and Capecchi, 1994). Similarly, activation or inhibition of the paralogous Hox B3 gene, which is also expressed in EC, modifies expression of several other Hox genes including D3 (Faiella et al., 1994). Nonetheless, in EC, expression of Hox D3 is sufficient to convert quiescent EC to an angiogenic/invasive state. This is supported by the observation that Hox D3 antisense prevents bFGF-mediated expression of both β3 integrin and uPA in these cells. Our findings also reveal that temporally restricted expression of Hox D3 is required for normal angiogenesis since prolonged expression of Hox D3 maintains the angiogenic/invasive EC phenotype, thereby disrupting normal vascular remodeling.

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