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Involvement of Endothelial CD44 during *in Vivo* Angiogenesis

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CD44, a cell-surface receptor for hyaluronan, has been implicated in endothelial cell functions, but its role in the formation of blood vessels *in vivo* has not been established. In CD44-null mice, vascularization of Matrigel implants and tumor and wound angiogenesis were inhibited. Leukocyte accumulation during tumor growth and wound healing in wild-type and CD44-null mice were comparable, and reconstitution of CD44-null mice with wild-type bone marrow did not restore the wild-type phenotype, suggesting that impairments in angiogenesis in CD44-deficient mice are due to the loss of endothelial CD44. Although the cell proliferation, survival, and wound-induced migration of CD44-null endothelial cells were intact, these cells were impaired in their *in vitro* ability to form tubes. Nascent vessels in Matrigel implants from CD44-null mice demonstrated irregular luminal surfaces characterized by retracted cells and thinned endothelia. Further, an anti-CD44 antibody that disrupted *in vitro* tube formation induced hemorrhage around Matrigel implants, suggesting that antagonism of endothelial CD44 undermined the integrity of the endothelium of nascent vessels. These data establish a role for CD44 during *in vivo* angiogenesis and suggest that CD44 may contribute to the organization and/or stability of developing endothelial tubular networks. ***(Am J Pathol 2006, 169:325–336; DOI: 10.2353/ajpath.2006.060206)***

Hyaluronan (HA), an important glycosaminoglycan constituent of the extracellular matrix, is composed of repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine.[1] This widely distributed molecule regulates cellular events such as cell proliferation and locomotion that are required for a variety of biological processes, including morphogenesis, tumorigenesis, inflammation, and host responses to injury (reviewed in Ref. 2). HA has also been implicated in the formation of vessels, but its effects on *in vivo* angiogenesis and endothelial cell (EC) function are complex and depend on HA concentration and molecular size.[3] High molecular weight HA (at concentrations of >100 μg/ml) inhibits EC proliferation and disrupts confluent endothelial monolayers.[4] These findings are consistent with the fact that avascular regions in chick embryo limb buds are rich in native high molecular weight HA and that expression of this form of HA in normally vascular areas results in decreased vascularity.[5] In contrast, low molecular weight HA stimulates EC proliferation,[4,6] wound-induced migration,[6] in vitro endothelial tube formation,[7] and neovascularization in chick chorioallantoic membranes[8] and cutaneous wounds.[9,10]

HA mediates its biological effects through binding interactions with specific cell-associated receptors.[11] A number of HA-binding proteins (so-called hyaladherins) have been identified, with CD44 and Receptor for HA-Mediated Motility (RHAMM) being the two best characterized cell-surface receptors for HA.[2] Although several other binding interactions for CD44 and RHAMM have been reported,[12,13] currently their interactions with HA appear to be the ones most likely to directly activate intracellular signals required to stimulate processes relevant to angiogenesis.[14] With respect to EC functions, previous studies have implicated CD44 in EC proliferation, migration, and adhesion to HA; RHAMM in EC motility; and both receptors in EC tube formation.[15–22]

Although there is evidence for the activity of RHAMM during *in vivo* angiogenesis,[16] the involvement of CD44 in the formation of blood vessels has not been estab-

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lished.[16] We therefore investigated *in vivo* angiogenesis in CD44-null mice and found that vascularization of subcutaneous Matrigel (Collaborative Biomedical Products, Bedford, MA) plugs, as well as tumor and wound angiogenesis, was inhibited in CD44-null animals. Leukocyte recruitment during tumor growth and wound healing in wild-type and CD44-null mice were similar, and reconstitution of CD44-deficient mice with wild-type bone marrow did not restore the wild-type phenotype, suggesting that impairments in angiogenesis in CD44-null mice result from the absence of endothelial and not leukocyte CD44. ECs were isolated from wild-type and CD44-null mice. Although the cell proliferation, survival, and wound-induced migration of the CD44-null ECs were intact, these cells were impaired in their *in vitro* ability to form tubes on Matrigel as compared to wild-type EC controls. Electron microscopic analysis of Matrigel implants in the CD44-null mice revealed nascent vessels with irregular luminal surfaces characterized by retracted cells and thinned endothelia. Treatment of wild-type mice with an anti-CD44 antibody that disrupted *in vitro* tube formation induced vessel hemorrhage around subcutaneous Matrigel implants, suggesting that antagonism of endothelial CD44 undermined the integrity of the endothelium of nascent vessels. These data establish for the first time the involvement CD44 in the *in vivo* formation of blood vessels and suggest that CD44 may be involved in morphological events required for the organization and/or stability of endothelial tubular networks during *in vivo* angiogenesis.

Materials and Methods

Reagents and Chemicals

All reagents and chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Antibodies

The following antibodies against murine surface receptors were used: IM7.8.1,[22] a rat anti-CD44 antibody from American Type Culture Collection (Rockville, MD); 390, rat anti-PECAM-1 antibody,[23] and F4/80 antibody against murine macrophages (Serotec, Raleigh, NC); anti-ICAM-2 antibody (Southern Biotech, Birmingham, AL); anti-murine CD11b antibody (Chemicon, Temecula, CA) and anti-CD8 and anti-CD45 antibodies (BD Pharmingen, San Jose, CA). Cell-surface antibody binding was determined by fluorescence-activated cell sorting (FACS) analysis using previously described procedures.[16]

Cell Lines

The H5V murine EC line,[23] B16 murine melanoma line (obtained from the American Type Culture Collection) and ID8-VEGF (vascular endothelial growth factor) tumor line, developed in the laboratory of Dr. George Coukos,[24] were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin/streptomycin, and 2 mmol/L L-glutamine, with insulin also added to the medium for the culturing of the ID8-VEGF cells. Lung ECs were isolated immunomagnetically from wild-type and CD44-null mice using published protocols[25] and were cultured in EGM-2 MV medium from Cambrex Bioscience Inc. (Walkersville, MD).

Animals

The Institutional Animal Care and Utilization Committees at both the Wistar Institute and the University of Pennsylvania School of Medicine approved all animal care procedures. CD44-null mice, on a C57BL/6 background, were the kind gift of Dr. Tak Mak (University of Toronto, Toronto, ON, Canada). Wild-type mice, also on a C57BL/6 background, were obtained from Taconic (Germantown, NY).

Matrigel Neovascularization Model

Wild-type and CD44-null mice in the C57BL/6 background were injected subcutaneously with 0.5 ml of Matrigel containing 1×10^6 B16 melanoma cells to induce the growth of vessels into the gel. After 5 to 7 days, the animals were sacrificed, and the gels were harvested and processed for hemoglobin analysis as a measure of the vascularization the gels. For the antibody studies, four injections (200 μg) of antibody were administered via the tail vein, beginning 2 days after Matrigel implantation.

Tumor Growth and Angiogenesis

2×10^6 B16 melanoma or ID8-VEGF ovarian tumor cells in a total volume of 50 μl were injected into wild-type and CD44-null mice. After 14 days (B16 melanoma) or 9 weeks (ID8-VEGF ovarian tumor) the mice were sacrificed, and the tumors were harvested, measured, and processed for staining.

Murine Model of Wound-Induced Angiogenesis

Wounding was performed as previously described.[26] Briefly, 1-cm^2 wounds were made in the skin on the upper back of the mice with the wounds placed so as to not injure the underlying muscle. Digital images of the wounds were captured at the time of wounding and then on days 1, 3, 5, 7, and 10 after placement of the wounds. At these time points the wounds and surrounding tissue were harvested and processed for immunohistochemical staining for vessel density and leukocyte infiltration.

Generation of Bone Marrow Chimeric Animals

Bone marrow chimeric mice were generated as previously described.[27] Briefly, to generate recipient animals, 6-week-old wild-type or CD44-null mice were irradiated with 1000 rads from a Cs-137 irradiation source. Within 24 hours after irradiation, donor marrow was obtained from the femur and tibia of non-irradiated mice and 5×10^6 cells were injected via the tail vein into the irradiated

recipient mice. Experiments were subsequently conducted 4 to 6 weeks after transplantation. FACS analysis of leukocytes using an anti-mouse CD44 antibody confirmed the phenotype of each chimeric mouse.

Immunohistochemical Staining

Immunohistochemistry was performed using a commercially available kit according to the manufacturer's instructions (ABC Immunodetection kit, Vector Laboratories, Burlingame, CA). Briefly, 6-μm-thick sections were prepared by cryostat, transferred to glass slides, and fixed in ice-cold acetone and rinsed in phosphate-buffered saline (PBS). The sections were then permeabilized with 0.3% Triton X-100 in PBS for 10 minutes, treated with 0.5% H_2O_2 in PBS for 30 minutes, and then blocked with 0.5% bovine serum albumin for 30 minutes. The sections were incubated with primary antibody for 1 hour, washed, and then incubated with biotinylated secondary antibody for 1 hour. The reaction was developed with an avidin-biotin complex reaction, and the sections were lightly counterstained with hematoxylin.

Quantitation of Angiogenesis and Leukocyte Accumulation

For quantitation of angiogenesis, frozen tissue sections were stained with monoclonal antibody 390 or ICAM-2 antibodies to identify murine blood vessels. To assess the tumor angiogenic response, serial sections were obtained from different levels within the tumor separated by ~100 μm. A total of four to eight levels per tumor were analyzed. For the B16 tumors, the vessel density at the margins of the tumors was determined by counting the number of vessels per 40× field. For the ID8-VEGF tumors, computer-assisted image analysis was used to determine the vessel area of a defined optical field of 3.6×10^5 μm^2 positioned at serial locations along the margins of the tumor. For each section eight to 12 optical fields were analyzed. To assess wound angiogenesis computer-assisted image analysis was used to determine the vessel density within 300 μm of the edge of the wound, expressed as the percentage of tissue at wound edge occupied by vessels. Staining for leukocytes and macrophages in wounded tissue was achieved with anti-CD11b and F4/80 antibodies, respectively, and the number of cells/40× field at the base of the wound was determined. FACS analysis of cells from minced and filtered tumors was used to determine the percentage of cells expressing CD8, CD11b, or CD45 leukocyte markers.

Light and Electron Microscopy

Tissues were fixed in 10% formalin (for paraffin sections) or 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) (for electron microscopy). Paraffin blocks were sectioned for hematoxylin and eosin staining, while electron microscopy sections were obtained after fixation in 2% osmium tetroxide and embedding in epoxy resin. Sufficient sections for representative serial sampling were produced and reviewed.

In Vitro *Cell Proliferation*

Endothelial cells were cultured for 24 hours in 96-well plates (4000 cells/well), and the number of viable cells was determined using the Cell Titer 96® A_{queous} Non-Radioactive Cell Proliferation Assay (Promega Madison, WI). This assay is composed of solutions of a novel tetrazolium compound (designated MTS) and an electron coupling (phenazine methosulfate) agent. MTS is bioreduced by dehydrogenase enzymes in metabolically active cells into a soluble formazan product. The absorbance of the formazan at 490 nm can be measured directly from 96-well assay plates without further processing and is directly proportional to the number of living cells in culture. As per the manufacture, the linear range for this assay is between 1000 and 200,000/well. In our hands we found a similar linear range for human umbilical vein endothelial cells with this assay (data not shown). Cell doubling was determined for isolated murine ECs by culturing the cells in T-25 flasks (25,000 cells/flask initially) over 6 days with the number of cells determined by Coulter counter.

HA Binding

Wild-type and CD44-null ECs were incubated with HA (Healon; Pharmacia and Upjohn, Kalamazoo, MI) for 30 minutes at 4°C, then washed two times with PBS. The cells were then lysed, and HA binding was determined by enzyme-linked immunosorbent assay according to the manufacturer's instructions (Echelon Bioscience, Inc., Salt Lake City, UT). HA binding was also assessed by FACS analysis in which wild-type and CD44-null cells were incubated with biotinylated HA for 60 minutes at 4°C, followed by incubation with fluorescein-conjugated streptavidin (Vector Laboratories).

HA Adhesion Assay

96-well plates were coated with HA (as Healon) using previously described procedures,[16] and 40,000 H5V cells in 200 μl of serum-free Dulbecco's modified Eagle's medium were added to the plates for 30 minutes. After gentle washing, the number of adherent cells was determined using a commercially available nonradioactive colorimetric assay according to the manufacturer's instructions (Titer 96 A_{queous} Non-Radioactive Cell Proliferation Assay).

In Vitro *Wounding-Induced Migration Assay*

EC wounding was performed as previously described.[28] 20,000 ECs were added to 24-well tissue culture plates and allowed to grow to confluence. Linear defects were then made in the monolayer. The wounded culture was

washed with PBS and then incubated for 24 hours in medium (with 1% serum). Using computer-assisted image analysis, and the Image-Pro Plus program (Media Cybernetics, Silver Spring, MD), images were obtained immediately after wounding and 24 hours later, and then the distance migrated by cells at the wound edge was determined. For each condition three to five wounds were analyzed.

In Vitro *Tube Formation Assay*

In vitro tube formation was studied using previously described procedures.[23] Fifty microliters of Matrigel was added to each well of a 96-well plate and allowed to form a gel at 37°C for 30 minutes. Cells (20,000) in 200 μl of complete medium were subsequently added to each well, and the mixture was incubated for 6 hours at 37°C in 5% CO_2. The wells were washed, and the gel and its cells were fixed with 3% paraformaldehyde. Total tube length per well was determined by computer-assisted image analysis using the Image-Pro Plus program.

In Vitro *Cell Death Detection*

For the studies of apoptosis, confluent cells were exposed for 5 hours to serum-free medium or complete medium, with or without antibody. Apoptosis was then assessed using the APO*Percentage* Apoptosis Assay (Bicolor Ltd., Belfast, Ireland).

Statistical Analyses

Differences among groups were analyzed using one-way analysis of variance. Results are presented as means ± SE. When statistically significant differences were found ($P < 0.05$), individual comparisons were made using the Bonferroni/Dunn test.

Results

Reduced Vascularization of Matrigel Implants in CD44-Null Mice

To investigate the involvement of CD44 in the formation of blood vessels, *in vivo* angiogenesis was studied in mice deficient in CD44.[29] Initial studies were performed with a model in which vessels develop around and within subcutaneously implanted Matrigel plugs containing B16 tumor cells as a source of angiogenic growth factors. After 5 to 7 days, a "blush" of vessel proliferation was seen surrounding plugs in the wild-type animals that was not evident in the CD44-null mice (Figure 1, A and B). Consistent with this, vascularization of the plugs, as assessed by hemoglobin concentration, was significantly reduced in CD44-deficient mice compared to wild-type animals (Figure 1C).

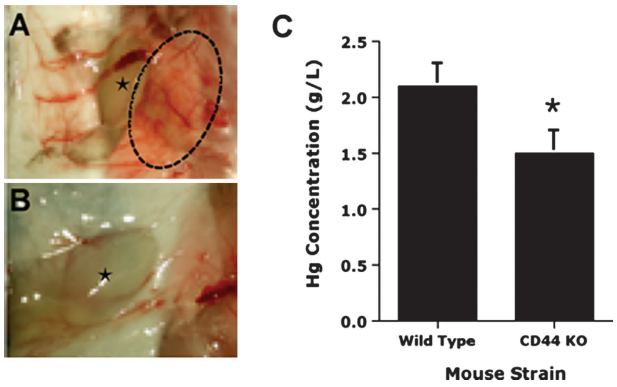


Figure 1. Neovascularization of Matrigel implants in wild-type and CD44-null mice. Shown are images of Matrigel implants (**star**) containing B16 melanoma cells as a source of angiogenic factors harvested after 5 days from wild-type (**A**) and CD44-null (CD44 KO) (**B**) animals. A "blush" of vessel proliferation (circle) was evident around the implants from the wild-type animal that was not present in the CD44-null mice. The vascularization of the gels, as assessed by hemoglobin concentration (**C**), was significantly reduced in CD44-null mice compared to wild-type mice. Data are presented as means ± SE ($n = 20$, $^*P = 0.02$).

Decreased Tumor Growth and Angiogenesis in CD44-Null Mice

To confirm and extend the findings of the Matrigel studies, the subcutaneous growth and associated tumor angiogenesis of the B16 melanoma line and an ovarian tumor line overexpressing VEGF (ID8-VEGF) were studied in CD44-deficient mice. The growth of both tumors in the CD44-null mice was significantly reduced (>70%) compared to that of tumors in wild-type animals (Figure 2, A, B, C, and E). The vessel densities of tumors of comparable sizes from wild-type and mutant mice were subsequently determined and found to be significantly reduced in the CD44-null mice (Figure 2, D and F). Further, histological analysis of B16 melanoma tumors from CD44-null animals revealed a paucity of the large sinusoidal vessels characteristic of the tumors harvested from wild-type mice (Figure 2G). These data suggest that the inhibition of tumor growth in the CD44-null mice resulted at least in part from a reduced angiogenic response.

Delayed Wound Healing with Reduced Angiogenesis in CD44-Null Mice

With the data above implicating CD44 in the pathological formation of blood vessels additional studies were performed to determine whether CD44 might also be involved in physiological angiogenesis. The closure of 1-cm^2 skin wounds was therefore studied in wild-type and CD44-null mice. We observed that wound closure in the CD44-mutant animals was delayed during days 1 to 3 after wounding but by day 7 had recovered and was similar to that of wild-type animals (Figure 3A). The density of vessels at the edge of the wounds on day 3 was reduced by 20% in the CD44-null animals compared to wild-type mice (Figure 3B). Thus, the absence of CD44 results in an early delay in the closure of skin wounds that is associated with a modest but significant reduction in the neovascularization of the wounded tissue.

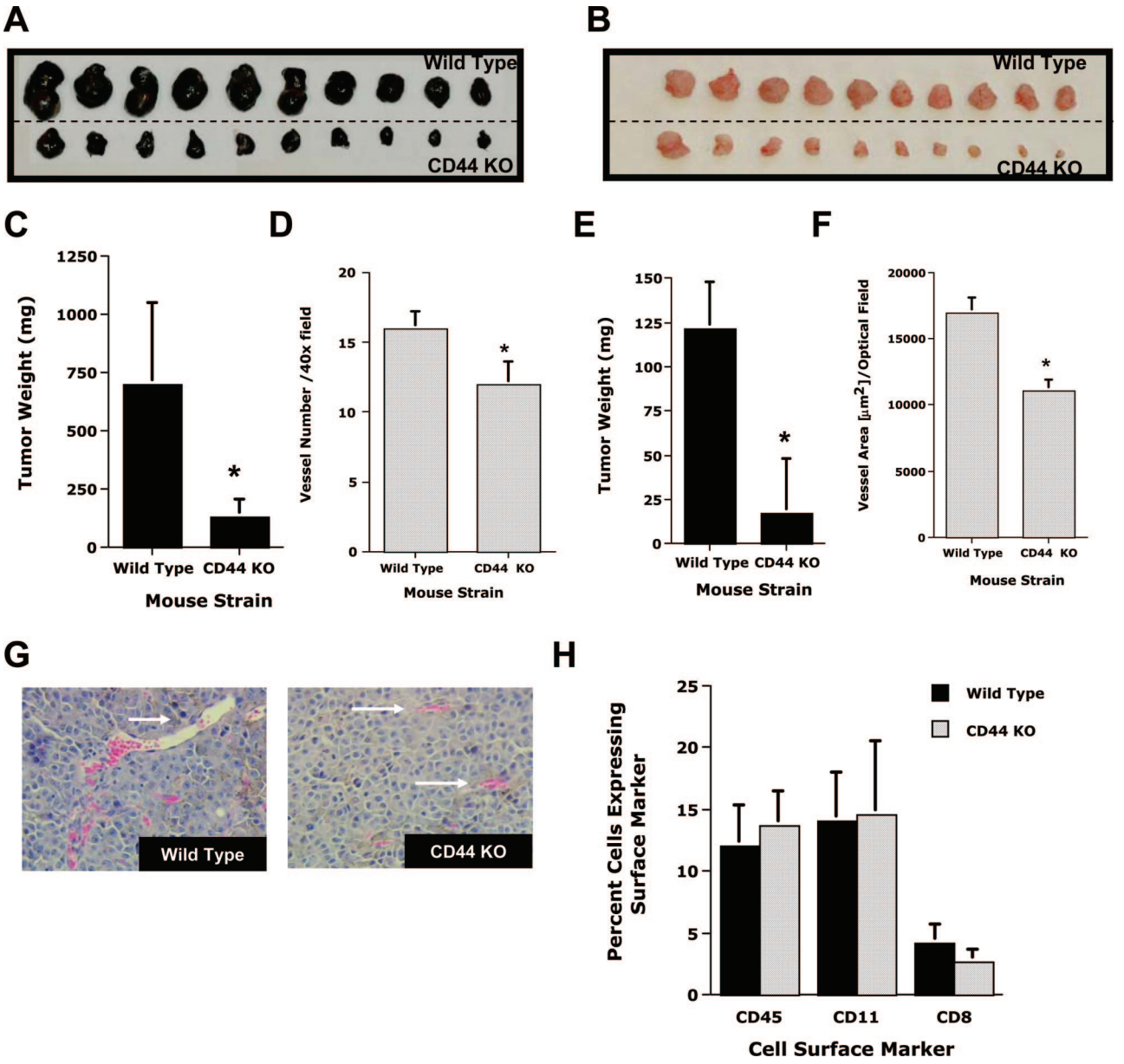


Figure 2. Tumor growth and angiogenesis in CD44-null mice. The growth of the B16 melanoma line (**A** and **C**) and the ID8-VEGF ovarian tumor line (**B** and **E**), as accessed by tumor weight, were significantly inhibited in the CD44-null (CD44 KO) mice ($n = 10$, $^*P < 0.01$). The vessel densities of tumors of comparable sizes, as assessed by vessel number/40× field for the B16 tumor (**D**) and vessel area, μm^2/optical field for the ID8-VEGF tumor (**F**), were significantly reduced in the CD44-null mice ($n = 4–6$, $^*P < 0.002$). **G:** Hematoxylin and eosin sections of B16 melanomas from CD44-null mice revealed a paucity of the large vascular spaces (**arrows**) that were readily observable in the wild-type animals. **H:** The percentage of cells from B16 tumors expressing leukocyte surface markers (CD11b, CD45, or CD8) was determined by FACS analysis and found to be comparable in wild-type and CD44-null animals. Data are presented as means ± SE.

Intact Leukocyte Recruitment during Tumor Growth and Wound Healing in CD44-Null Mice

HA receptors are expressed on leukocytes and have been implicated in leukocyte trafficking.[30–32] Consequently, the absence of CD44 could lead to less leukocyte recruitment and thus a loss of a source of pro-angiogenic factors. We, however, found that leukocyte accumulation in wild-type and CD44-mutant mice in the B16 and ID8-VEGF tumors (Figure 2H; data not shown for ID8-VEGF tumors) and during wound healing were comparable (Figure 3C). This suggests that the diminished tumor and wound angiogenesis arising from the loss of CD44 was not the result of reduced leukocyte recruitment.

Murine Angiogenesis Involves Endothelial CD44

The recruitment data presented above do not exclude functional consequences arising from the loss of CD44 expression on leukocytes or on circulating bone marrow-derived progenitor endothelial cells, both of which have been identified along with ECs as cellular participants during *in vivo* angiogenesis.[33–38] To identify the possible involvement of CD44 expressed on recruited leukocytes,

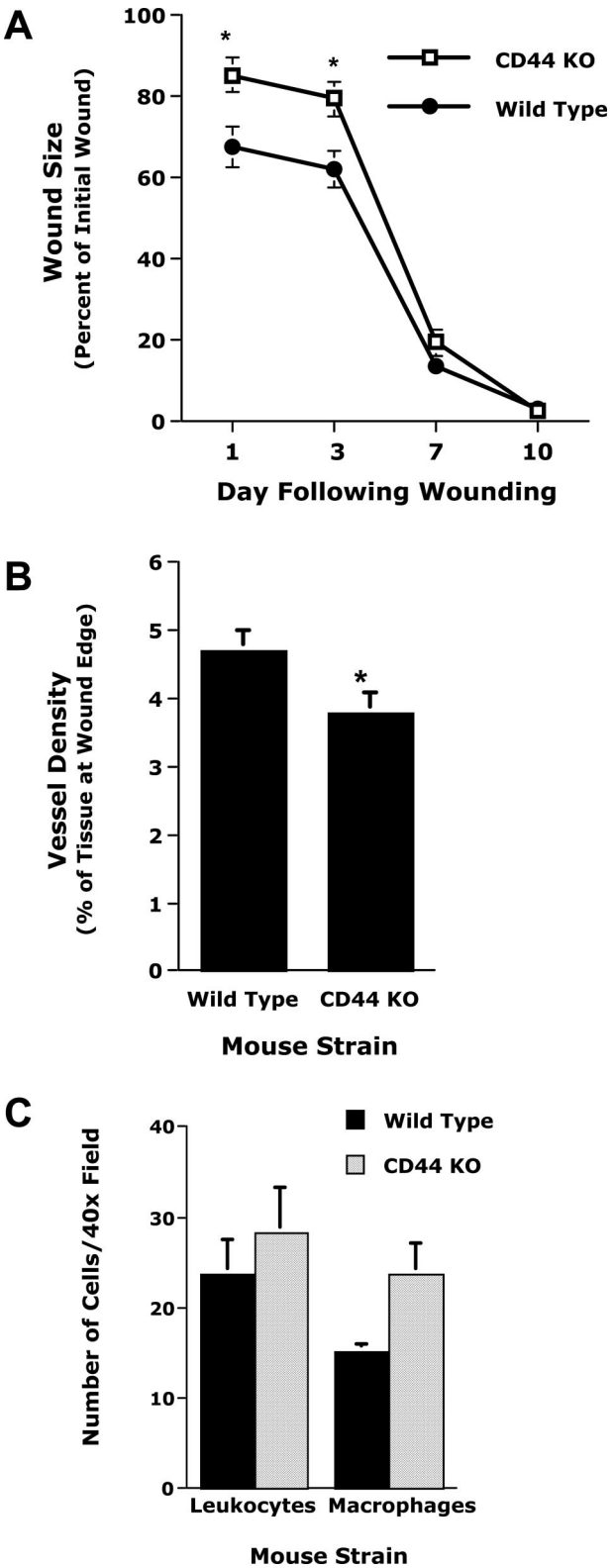


Figure 3. Wound healing and angiogenesis in CD44-null mice. **A:** The closure of 1-cm^2 skin wounds, as measured by percentage of initial wound, was less at days 1 and 3 after wounding in CD44-null (CD44 KO) animals compared to wild-type mice, but not at later time points ($n = 6$, $^*P < 0.02$). **B:** Vessel density at the edge of the wound (percentage of tissue at wound edge occupied by vessels) on day 3 was reduced (by 20%) in the CD44-null mice (**B**) ($n = 8$, $^*P < 0.03$). **C:** Leukocyte and macrophage accumulations (number of cells/40× field) in the two mice strains were comparable ($n = 4$; $^*P > 0.1$). Data are presented as means ± SE.

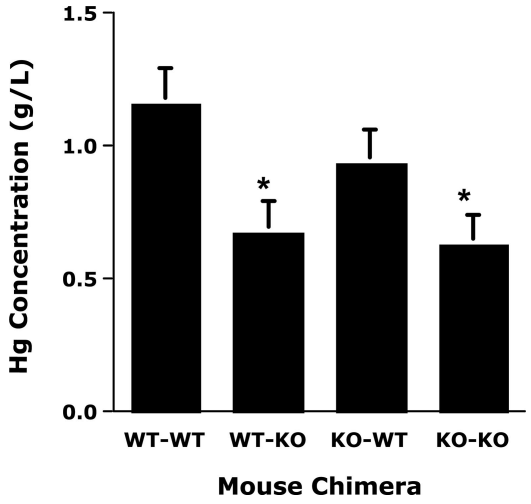


Figure 4. Neovascularization of Matrigel implants in bone marrow chimeric mice. Vascularization of Matrigel implants was studied in the following chimeric (donor-recipient) mice: wild-type into wild-type mice (WT-WT), wild-type into CD44-null mice (WT-KO), CD44-null into wild-type mice (KO-WT), and CD44-null into CD44-null mice (KO-KO). The angiogenic responses (assessed by hemoglobin concentration) in the WT-KO and KO-KO mice were very similar but were significantly reduced compared to the WT-WT and KO-WT responses, which were similar. Data are presented as means ± SE ($n = 8$, $^*P < 0.02$).

tissue macrophages, and/or circulating endothelial progenitor cells in vessel formation, bone marrow chimeric animals were generated to selectively propagate bone marrow-derived wild-type vascular cells against a background of CD44-deficient endothelium. Experiments were performed in which the following chimeric (donor-recipient) mice were generated: wild-type into wild-type mice (WT-WT; wild-type control), wild-type into CD44-null mice (WT-KO), CD44-null into wild-type mice (KO-WT), and CD44-null into CD44-null mice (KO-KO; null control). FACS analysis confirmed that the blood leukocytes from the WT-WT and WT-KO mice expressed CD44, whereas the leukocytes from the KO-WT and KO-KO animals were devoid of CD44 (data not shown). The vascularization of subcutaneous implanted Matrigel plugs was subsequently studied (Figure 4). The angiogenic responses in the WT-KO and KO-KO mice were comparable but were significantly reduced compared to the WT-WT and KO-WT responses, which were similar to each other. The inability of wild-type CD44-expressing leukocytes and/or bone marrow-derived endothelial progenitor cells to restore the wild-type phenotype in the null animals (WT-KO) and the failure of the corresponding CD44-null cells to induce an impaired angiogenic response (KO-WT) are consistent with the primary involvement of endothelial CD44 during *in vivo* angiogenesis.

CD44-Null ECs Demonstrate Preserved HA Adhesion, Proliferation, Migration, and Susceptibility to Apoptotic Stress but Impaired Tube Formation on Matrigel

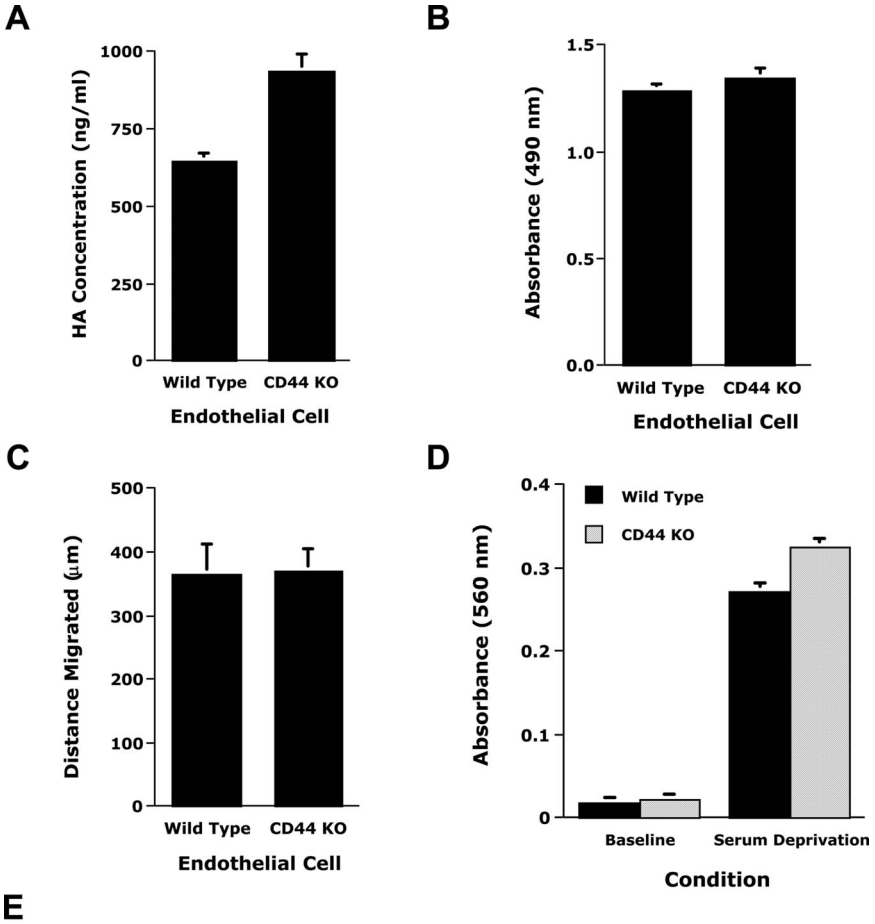
The studies of the bone marrow chimeric animals suggested that the loss of endothelial CD44 function inhib-

its *in vivo* angiogenesis. To further investigate this, the activities of ECs isolated from wild-type and CD44-null animals were studied. Although CD44 has long been recognized as a major surface receptor for the binding of HA,[11,12] we found that HA binding to CD44-null ECs was preserved and actually may have been greater than that of wild-type EC (Figure 5A). These findings were confirmed by FACS analysis (Table 1), although the overall level of HA binding was low. Cell proliferation for the two cell types was similar whether assessed by a colorimetric assay (Figure 5B) or by the number of cell doublings over 6 days (6.8 ± 0.2 for WT EC vs. 6.7 ± 0.1 for CD44-null EC). Further, motility in wild-type and CD44-deficient ECs was comparable, and both cell types were similarly susceptible to serum deprivation-induced apoptosis (Figure 5, C and D). Studies of *in vitro* tube formation on Matrigel, however, demonstrated that the loss of CD44 severely compromised the ability of the CD44-null ECs to form tubular networks on this substrate (Figure 5E).

Table 1. HA Binding to Murine ECs Assessed by FACS Analysis

	Background	Wild-type EC	CD44-null EC
Mean fluorescence intensity	0.217	0.315	0.424

Murine ECs isolated from wild-type and CD44-null mice were treated with biotinylated HA, incubated with fluorescein-conjugated streptavidin, and then subjected to FACS analysis. Shown are the mean fluorescence intensities. Background staining is that of WT cells stained only with secondary antibody.



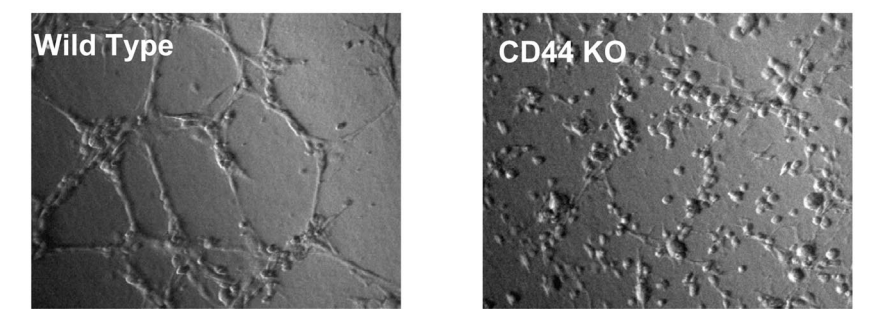


Figure 5. *In vitro* function of murine ECs isolated from wild-type and CD44-null mice. **A:** Binding of HA to ECs isolated from wild-type and CD44-null (CD44 KO) mice was determined by enzyme-linked immunosorbent assay (n = 2). Before the addition of exogenous HA, the background levels of HA associated with cell surface of the wild-type and the CD44-null cells were 384 and 295 ng/ml, respectively. HA binding to CD44-null cells was preserved. **B:** The proliferation of wild-type and CD44-null ECs cultured for 24 hours in the presence of serum was assessed using a colorimetric assay and measurement of the reaction mixture at 490 nm. The proliferative responses of the two cell types were comparable (n = 4). **C:** Liner defects were made in confluent cell monolayers, and closure of the wounds after 24 hours was assessed by computer-assisted image analysis (n = 12). Wound-induced migration was similar in the two cell types. **D:** Apoptosis was assessed after 5 hours in the presence or absence of serum. Wild-type and CD44 KO ECs were comparable in their susceptibility to serum deprivation (n = 15). **E:** Shown are representative images of wild-type and CD44-null ECs plated on Matrigel, demonstrating impaired tube formation by the CD44-null ECs.

Electron Microscopic Analysis of Actively Forming Vessels Reveals Morphologically Different Microvessels in CD44-Null Mice

The impairment of *in vitro* tube formation by CD44-null ECs led to further investigations of whether the structure of actively forming vessels might be affected by the absence of CD44. Electron microscopic analyses were therefore performed on the vessels invading subcutaneous Matrigel implants harvested from wild-type and CD44-deficient mice (Figure 6). Compared to the wild-type animals, the endothelia of these invading vessels in the CD44-null mice were characterized by an absence of cellular ruffling and an irregular surface punctuated by retracted cells and/or very thin or flattened endothelial cells. Further, numerous pinocytotic vesicles were readily detected in the microvessels of the wild-type capillaries but were less evident in the CD44-null vessels. Together these data suggest that the assembly, organization, and/or maturation of the endothelium of actively forming vessels are altered by the loss of CD44.

Anti-CD44 Antibody (IM7.8.1) Inhibits Tube Formation and Induces Hemorrhage around Matrigel Implants in Wild-Type Mice

In prior studies, neovascularization of Matrigel implants was not inhibited by KM81, an anti-CD44 antibody.[16] Our finding that the angiogenic response in the Matrigel assay was decreased in the CD44-null mice, led us to re-explore the effects of acute antibody inhibition by studying the activity of another well characterized anti-CD44 antibody, IM7.8.1.[22] The specificity of this antibody was confirmed by the fact it bound to wild-type but not CD44-null ECs (data not shown). IM7.8.1 inhibited the binding of murine ECs to HA (Figure 7A) but had no effects on cell proliferation, migration, or susceptibility to apoptotic stress (Figure 7, B–D). *In vitro* tube formation on Matrigel, however, was significantly inhibited by IM7.8.1 (Figure 7E). Further, grossly visible hemorrhage was observed around the implants of mice treated with IM7.8.1 but not in animals treated with a control antibody (Figure 8). The antibody did not induce thrombocytopenia or hemorrhage in other organs (data not shown). Given this, and the fact that the antibody inhibits *in vitro* tube formation, these *in vivo* data suggest that the acute inhibition of endothelial CD44 function during the active formation of vessels may undermine the integrity of these nascent vessels, making them more likely to rupture.

Discussion

In this report, studies were performed to define the *in vivo* involvement of CD44 in blood vessel formation. We found that vascularization of Matrigel implants as well as tumor and wound angiogenesis were inhibited in CD44-null mice. Leukocyte accumulation during tumor growth and wound healing were comparable in wild-type and CD44-null animals, suggesting that impairments in leukocyte recruitment did not contribute to the angiogenic phenotype of the CD44-null mice. Reciprocal bone marrow transplants involving wild-type and CD44-deficient mice demonstrated that the impaired angiogenic response resulted from a loss of endothelial, not leukocyte, CD44. In subsequent studies of ECs isolated from these animals, we found that *in vitro* tube formation was significantly compromised in the ECs isolated from CD44-deficient mice. Consistent with these *in vitro* data, the nascent vessels of Matrigel implants from CD44-null mice exhibited abnormal morphology. Lastly, hemorrhage developed around Matrigel implants treated with anti-CD44 antibody that inhibits *in vitro* tube formation, suggesting that inhibition of CD44 function may undermine the stability of newly formed vessels. Together these data provide evidence of the involvement of endothelial CD44 in the formation of vessels, possibly by mediating morphological events required for the organization and/or stability of endothelial tubular networks.

CD44 is a type 1 transmembrane glycoprotein that is expressed by most cell types, including ECs,[16] and is considered the major cell-surface receptor for HA.[2,11,12] With respect to the molecule's interaction with low molecular weight HA, studies using HA oligomers of defined sizes have demonstrated that oligomers of six sugars are the minimal size fragments required for monovalent binding to CD44, with oligomers bearing 20 or more sugars having the apparent capacity for divalent binding.[39] This multifunctional transmembrane receptor is capable of inducing signal transduction pathways[14] and is involved in cell-cell and cell-matrix interactions that mediate anchoring to HA-rich pericellular matrices, internalization of HA, cell locomotion, growth factor activation, leukocyte recruitment, and lymphocyte homing and activation.[12] This diversity of function arises from a large number of isoforms generated by alternative splicing and their subsequent variable glycosylation.[11,12]

For a growing tumor or wounded tissue, the local "angiogenic" environment is one that is characterized by increased expression of hyaluronidases,[40–43] and oxidative and nitrative stresses,[44] factors known to promote the degradation of high molecular weight species of HA into pro-angiogenic lower molecular weight fragments. It has been presumed that CD44 mediates HA-dependent cellular events involved in regulating EC functions required for angiogenesis. In line with this are studies of human or bovine ECs that implicate CD44 in HA-dependent adhesion, proliferation, migration, and/or tube formation.[15–22] Somewhat surprisingly, however, treatment of mice with KM81, a bioactive anti-murine CD44 antibody, failed to consistently inhibit the neovascularization of Matrigel implants containing basic fibroblast growth factor as the angiogenic factor.[16] These results may have been related to the assay used, a possible lack of activity of KM81 against EC functions and/or compensation by RHAMM, and thus the question of CD44's involvement during *in vivo* angiogenesis remained an open one. In light of this, our findings are important because they provide, for the first time, clear evidence of the activity of CD44 during *in vivo* angiogenesis in the adult animal (Figure 1), including both pathological (Figure 2) and

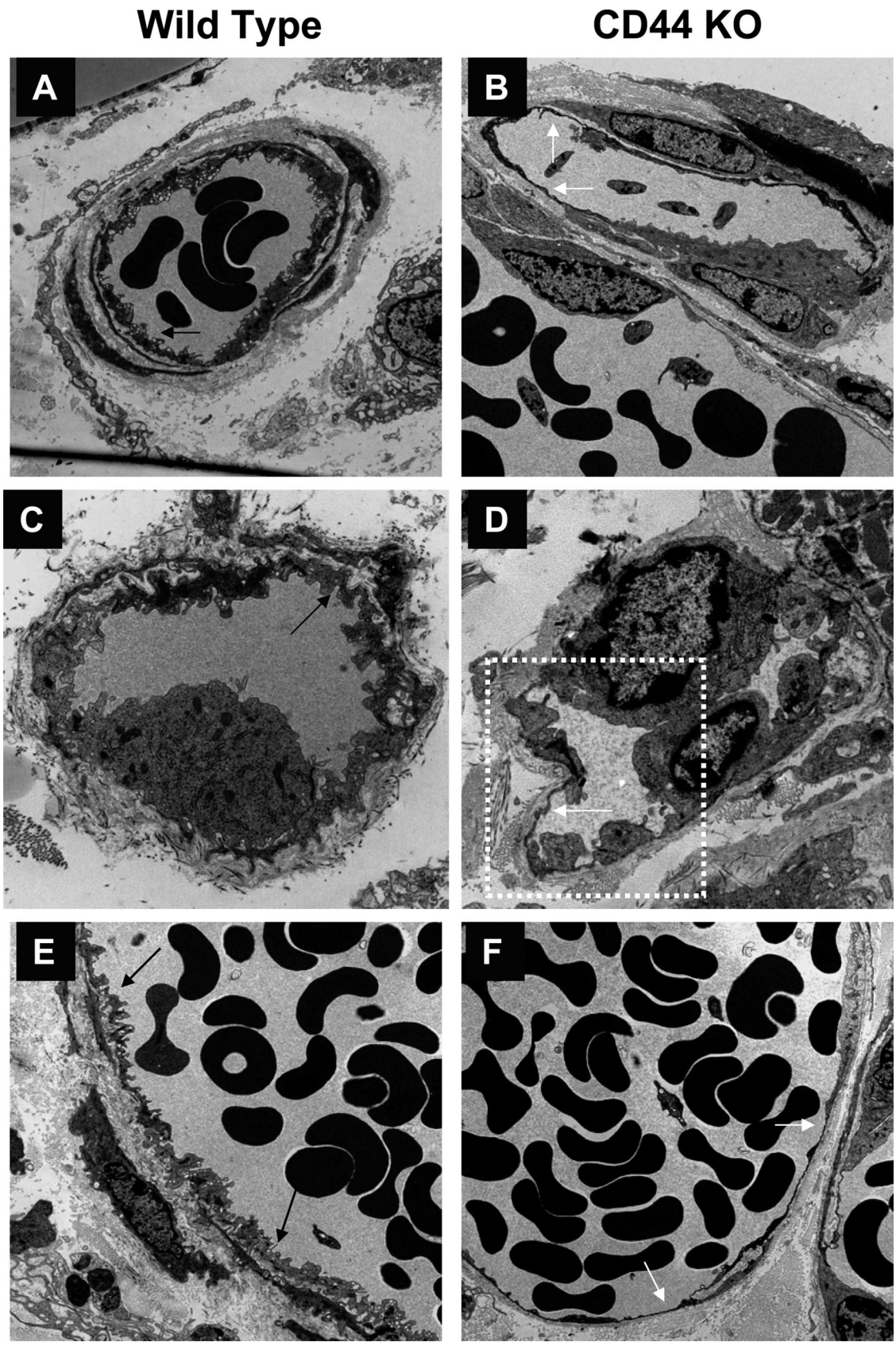


Figure 6. Electron microscopic analysis of actively forming vessels. Shown are electron micrographs of representative vessels invading subcutaneous Matrigel implants from wild-type (**A**, **C**, and **E**) and CD44-null mice (**B**, **D**, and **F**). The endothelia of these invading vessels in the CD44-null mice were characterized by an absence of cellular ruffling (**black arrows**) and an often very irregular surface (evident in the **boxed area** of **B**) punctuated by very thin or flattened ECs (**white arrows**).

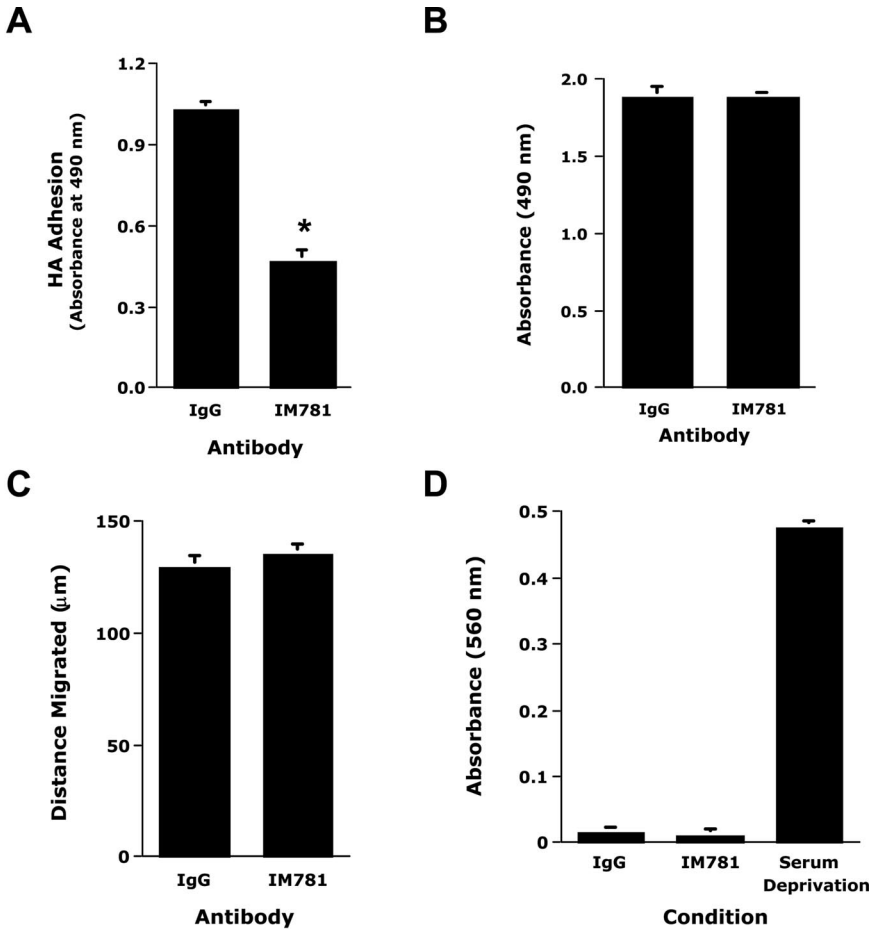


Figure 7. Effects of IM7.8.1 antibody on the *in vitro* function of murine ECs. Studies were performed of the effects of anti-CD44 antibody (IM7.8.1) on various functions of the H5V murine EC line. **A:** The adhesion of the H5V cells to HA-coated surfaces was inhibited by IM7.8.1 ($n = 4$, $^*P < 0.001$). **B:** The proliferation of H5V cells cultured for 24 hours in the presence of serum was assessed using a colorimetric assay and measurement of the reaction mixture at 490 nm. IM7.8.1 did not inhibit the proliferative response ($n = 4$). **C:** Linear defects were made in confluent cell monolayers, and closure of the wounds after 24 hours was assessed by computer-assisted image analysis ($n = 6$). Wound-induced migration was not inhibited by IM7.8.1. **D:** Apoptosis was assessed after 5 hours in the presence or absence of serum. Wild-type and CD44 KO ECs were comparable in their susceptibility to serum deprivation ($n = 24$). **E:** Shown are representative images of H5V cells plated on Matrigel that demonstrate that IM7.8.1 impairs tube formation.

physiological (Figure 3) settings. The fact, however, that CD44-null mice are viable, along with the absence of a perinatal cardiovascular phenotype, suggests that vascular development in the absence of CD44 is sufficient to permit adequate embryogenesis.[29] Whether this reflects compensation by RHAMM (or another cell-surface hyaladherin) will be addressed in future studies when RHAMM and CD44 double knockout mice are generated and analyzed.

CD44 is expressed not only on ECs but on bone marrow-derived cells such as recruited leukocytes, tissue macrophages, and circulating endothelial progenitor cells that also participate in angiogenesis.[33–38] The loss of CD44 expression on these bone marrow-derived cells, in addition to the absence of CD44 on ECs, could contribute to the angiogenic phenotype of the CD44 mutant mice. The inability of wild-type CD44-expressing leukocytes and/or bone marrow-derived endothelial progenitor cells to restore the wild-type phenotype in the null animals (WT-KO) and the failure of the corresponding CD44-null cells to induce an impaired angiogenic response in the wild-type mice (KO-WT) provide strong evidence for the endothelial involvement of CD44 during *in vivo* angiogenesis (Figure 4). Further, leukocyte recruitment into tumors (Figure 2H) or wounded tissue (Figure 3C) was preserved in CD44 mutant mice, suggesting that the diminished angiogenic response was not the result of compromised CD44-dependent leukocyte trafficking. Together, these data point to the importance of endothelial CD44, in contrast to leukocyte CD44, in the formation of blood vessels.

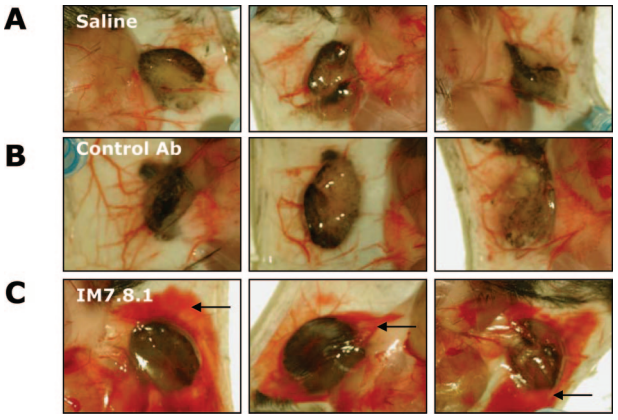


Figure 8. Effects of IM7.8.1 antibody on the neovascularization of Matrigel implants. Shown are representative images of Matrigel implants containing B16 melanoma cells as a source of angiogenic factors harvested after 5 days from animals injected with saline (**A**), a control antibody (**B**), and the IM7.8.1 antibody (**C**). Hemorrhage (**arrows**) was observed around the gels from animals treated with IM7.8.1 antibody.

With respect to the mechanism of action of CD44 during angiogenesis, our data demonstrating impairments in tube formation *in vitro* by CD44-null ECs (Figure 5E) or following treatment of murine ECs with anti-CD44 antibody (Figure 7E) provide evidence of a role for CD44 in the assembly of EC-lined channels. The abnormal morphology of the CD44-null neovessels in the Matrigel model (Figure 6) and the susceptibility of the wild-type form of these vessels to hemorrhage during treatment with anti-CD44 antibody (Figure 8) are consistent with these *in vitro* data and implicate CD44 in the organization and/or stabilization of the endothelia of forming or newly formed vessels. Whether these morphological differences are present in normal vessels during development is unknown but is currently under investigation.

Although the role of CD44 in the morphogenesis of endothelial tubes is still unknown, one or more processes may be involved. First, epithelial cell HA acts as a modulator of cell adhesion that mediates the early stages of the cell-substrate interaction.[45] If comparable processes exist for ECs, and are mediated by CD44, the loss of the expression of this molecule could disrupt initial adhesive events required for tube assembly. Second, CD44 through association with matrix metalloproteinases may promote remodeling of the endothelial pericellular matrix that is required for endothelial tube formation.[17,46] Third, certain isoforms of CD44 have heparan sulfate side chains that could provide for the attachment of heparin-binding angiogenic factors such as VEGF and basic fibroblast growth factor.[47] The binding of these factors to the endothelium by CD44 could facilitate their activity in the stabilization and maturation of newly formed vessels. Last, as newly formed vessels begin to mature, CD44 may locate and organize high molecular species in the basement membrane, which, given their inhibitory effects on EC proliferation and migration,[4,5] may induce vessel quiescence. These possible mechanisms are the subject of ongoing investigation.

In considering further the possible *in vivo* actions of endothelial CD44, two additional processes should be noted. First, although we found that the cell-proliferative responses of murine ECs appear to be independent of CD44 (Figures 5B and 7B), other studies have implicated CD44 in the proliferation of human umbilical vein endothelial cells.[15,16,21,22] Second, CD44 has been reported to be involved in the migration and/or invasion of human and bovine ECs through matrix-coated filters, apparently by mechanisms that involve activation of Rho GTPases and a cooperative interaction with the matrix metalloproteinase-9.[17,19,20] In contrast to this possible involvement of CD44 in cell motility, we found that loss of CD44 expression (Figure 5C) or antibody antagonism of its function (Figure 7B; see also Ref. 16) did not impair wound-induced migration. These differences in the requirement of CD44 for EC proliferation and migration may reflect species-dependent differential expression of endothelial CD44 isoforms. Also, the differences noted with respect to cell migration may reflect differences in the assays used, as published reports have used chemotaxis studies while we have relied on a model of wound-induced migration. Studies of single cell migration are ongoing to more clearly define the involvement of CD44 in endothelial cell motility using ECs isolated from wild-type and CD44-null animals. Thus, although our data implicate a role for CD44 in tube formation, it may also mediate EC proliferation and migration *in vivo*.

The finding that IM7.8.1 inhibited the adhesion of murine EC to HA (Figure 7A) is consistent with previous studies of human ECs,[16] as well as the fact that CD44 has long been considered the major cell-surface receptor for HA binding.[2,11,12] In contrast to this, we found that HA binding to CD44-null ECs was not reduced and in fact may have been increased (Figure 5A and Table 1). The preservation of HA binding suggests that other HA-binding receptors may be able to compensate for the loss of CD44-mediated adhesion. More importantly, however, these data suggest that there may well be aspects of CD44-dependent signaling that are either independent of HA[21] and/or cannot be reconstituted by HA interactions with other receptors.

In summary, the loss of CD44 impairs *in vivo* angiogenesis. This effect is due principally to the loss of expression of endothelial CD44, an effect that may be related to compromises in the ability of nascent vessels to properly assemble endothelium-lined tubes, although our data do not exclude impairments in EC proliferation and migration. Understanding its potential multiple roles in the formation of vessels may provide insights into CD44 as a therapeutic target.

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