Retinoic acid regulates endothelial cell proliferation during vasculogenesis

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Summary

A dietary deficiency of vitamin A is associated with cardiovascular abnormalities in avian and murine systems. Retinoic acid (RA) is the active metabolite of vitamin A and whether it directly regulates mammalian blood vessel formation has not been determined and is investigated herein. We used mice rendered RA-deficient via targeted deletion of retinaldehyde dehydrogenase 2 (Raldh2–/–), the enzyme required to produce active RA in the embryo. Histological examination at E8.0-8.5, prior to cardiac function and systemic blood circulation, revealed that capillary plexi formed in Raldh2–/– yolk sacs and embryos, but were dilated, and not appropriately remodeled or patterned. Raldh2–/– endothelial cells exhibited significantly increased expression of phosphohistone 3 and decreased expression of p21 and p27, suggesting that RA is required to control endothelial cell cycle progression during early vascular development. Uncontrolled endothelial cell growth, in Raldh2–/– mutants, was associated with decreased endothelial cell maturation, disrupted vascular plexus remodeling and lack of later stages of vessel assembly, including mural cell differentiation. Maternally administrated RA restored endothelial cell cycle control and vascular patterning. Thus, these data indicate that RA plays a crucial role in mammalian vascular development; it is required to control endothelial cell proliferation and vascular remodeling during vasculogenesis.

Introduction

The vasculature is the first organ system to develop during embryogenesis. Proper development of all other organ systems is dependent on the ability of the embryo to establish a circulatory network for the controlled distribution of nutrients and oxygen, and the removal of metabolic waste products from all tissues. Blood vessel formation begins in the yolk sac with the coalescence of mesodermal progenitors and their differentiation into endothelial and blood cells, forming blood islands (Doetschman et al., 1987) that fuse and define a primitive vascular plexus (Noden, 1989). Vascular induction within the mesoderm is thought to be mediated by bFGF (Risau et al., 1988a) and signaling molecules expressed in the adjacent extra-embryonic visceral endoderm, including Indian hedgehog (Ihh) (Dyer et al., 2001) and VEGFA (Miquerol et al., 2000).

The vascular plexus that initially forms does so in the absence of blood flow. However, extensive remodeling of the plexus into a branched circulatory network (Risau et al., 1988b) is dependent upon the onset of flow, suggesting that mechanical forces, as well as soluble factors within circulation are needed for normal vessel development. Vascular remodeling is a complex process that involves cell-cell (gap junctions) (Hirsch et al., 2003; Kruger et al., 2000) and cell-matrix (Francis et al., 2002) interactions, as well as the coordination of multiple signaling pathways. Signaling pathways implicated in this process include: TGFβ and TGFβRI and II, which regulate endothelial cell matrix production and maturation (Dickson et al., 1995; Larsson et al., 2001); ephrin B and Ephb receptors, which modulate cell-cell interactions and patterning (Gerety and Anderson, 2002; Oike et al., 2002); and angiopoietin 1 and the Tie2 receptor (Sato et al., 1995; Suri et al., 1996), as well as PDGFβ and PDGFRβ (Lindahl et al., 1997; Soriano, 1994), which mediate endothelial-induced mural cell (pericytes or smooth muscle cells) recruitment. Other pathways, including retinoid signaling, have been implicated in cardiovascular development; however, a cellular role for retinoids in mammalian blood vessel assembly has not been defined.

Retinoids are derivatives of Vitamin A (retinol) that exert a wide variety of profound effects on vertebrate development (DeLuca, 1991; Zile, 2001). Vitamin A is an essential nutrient, required for crucial biological functions in quantities that far exceed what can be metabolically generated. Thus, survival of
mammalian and avian species is dependent upon acquisition of adequate dietary vitamin A, which must be enzymatically converted to active retinoic acid (RA). The terminal step in RA synthesis is carried out by members of the class I aldehyde dehydrogenase (ALDH) family (Duester, 2000; McCaffrey and Drager, 2000; Ulven et al., 2000). Among these, retinaldehyde dehydrogenase 2 (Raldh2; Aldh1a2 – Mouse Genome Informatics) has high substrate specificity for retinaldehyde (Zhao et al., 1996). Embryos lacking this enzyme are RA deficient and die in utero at E10.5 (Niederreither et al., 1999).

The RA signal is transduced through two families of ligand dependent transcriptional regulators, RA receptors (RARs) and retinoid X receptors (RXR), which bind as heterodimers to DNA motifs (RA response elements, RAREs) and thus regulate the transcripational activity of target genes (reviewed by Mangelsdorf et al., 1994; Chambon, 1996). Nutritional vitamin A deficiency (VAD) in the rat (Wilson and Warkany, 1949), as well as RAR and RXR knockouts in the mouse, yield many defects, including abnormal ventricular trabeculation, defective outflow tract septation and aortic arch malformations (Mendelsohn et al., 1994; Sucov et al., 1994, Kastner et al., 1994; Kastner et al., 1997). Quail embryos that develop under full VAD display more severe heart defects and lack omphalomesenteric vessel formation (Heine et al., 1985).

Mice deficient for Raldh2 exhibit a similar phenotype as quail VAD embryos. Raldh2 is first expressed in the posterior mesoderm of the mouse embryo during gastrulation (Niederreither et al., 1997), and is expressed in the visceral endoderm at E7.5-8.5 (B.L.B., L.L., Pascal Dolle and K.K.H., unpublished) at the time of endodermal-mediated vascular induction in adjacent mesoderm. Importantly, retinoic acid receptors are expressed in endothelial cells in the adjacent mesoderm (B.L.B., L.L., Pascal Dolle and K.K.H., unpublished). Lack of Raldh2 expression in the yolk sac is correlated with disrupted formation of extra-embryonic vessels in Raldh2−/− mutants (Niederreither et al., 1999). However, Raldh2−/− embryos also exhibit defects in heart looping morphogenesis, and severely hypoplastic atria and sinus venosus development (Niederreither et al., 2001). Vascular defects can be the indirect result of cardiac malformations; therefore, we aimed to determine whether RA directly regulates vascular cell behavior and blood vessel formation using Raldh2−/− mice.

Histological examination revealed vascular malformations in RA-deficient yolk sacs and embryos prior to the onset of cardiac function and blood circulation. Cellular and molecular analyses indicated that RA was not required for endothelial cell differentiation, but was required for endothelial cell maturation and the control of cell cycle progression. Raldh2−/− mutants exhibited suppressed p21 and p27 induction, enhanced endothelial cell proliferation, and disrupted vascular plexus remodeling and patterning. Continuous RA supplementation of maternal diet restored cell cycle control and rescued the observed vascular defects in Raldh2−/− embryos and yolk sacs. Hence, RA plays a crucial role in mammalian vascular development, and is required throughout development to control endothelial cell growth and vascular remodeling. Such insight may be useful for the modulation of neovascularization during the progression of prevalent pathologies in which uncontrolled endothelial cell growth is a primary defect, or for the control of vessel assembly ex vivo in tissue engineering applications.

Materials and methods
Genotyping
The expression of the Raldh2 gene was disrupted in CD1 mice via targeted insertion of the neomycin gene into the coding region, as previously described (Niederreither et al., 1999). Heterozygote stocks were maintained by sibling mating; all offspring were genotyped using primers, which closely flank the Raldh2 locus, as described (Niederreither et al., 1999). The DNA source for genotyping was ear punches or tail clippings, which were digested in 200 µl 1×PCR buffer with 0.01 mg/ml protease K (Gibco BRL, Grand Island, NY) at 56°C for 3 hours followed by a 10 minute incubation at 96°C. Embryonic genotyping was performed using the same method, except that embryos or embryonic tails were used as template DNA.

Histology and Immunohistochemistry
Mouse embryos and yolk sacs (E8.25-9.5) were fixed in 4% paraformaldehyde for 2-3 hours; embedded in 3% agarose and then paraffin wax; sectioned, or quick-frozen in liquid nitrogen; embedded in OCT compound; and sectioned. Hematoxylin and Eosin staining of 5-10 µm paraffin wax sections of embryonic and yolk sac tissue was performed according to standard methods. Immunohistochemistry of 7-10 µm frozen sections was performed, as described (Novoseres et al., 2002), using the following primary antibodies (diluted in 4% NGS/3% BSA/PBS blocker), incubated for 2-4 hours at room temperature: rat anti-VE-cadherin (Pharmingen, San Diego, CA), 1:150 dilution; mouse anti-SM-α-actin (DAKO, Carpentrya, CA), 1:1000 dilution; mouse anti-p21, 1:200 (NeoMarkers, Freemont, CA); and mouse anti-p27, 1:200 (NeoMarkers). Species-specific biotinylated secondary antibodies were diluted 1:250 and incubated for 1 hour at room temperature. Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) with its corresponding peroxidase substrate Vector VIP were used, according to the manufacturer’s instructions, to reveal antibody-antigen complexes.

Embryos and yolk sacs at various stages of development (E8.25-12.5) were prepared for whole-mount immunohistochemistry, as described (Novoseres et al., 2002). Fixed tissues were rehydrated, permeabilized and incubated overnight at 4°C with one of the following primary antibodies (diluted in PBS containing 0.2% nonfat dry milk and 0.5% Triton X-100): mouse anti-SM-α-actin (DAKO), 1:1000; mouse anti-CD31, 1:100 (PE-CAM-1, Pharmingen); mouse anti-p21, 1:200 (NeoMarkers); and mouse anti-p27, 1:200 (NeoMarkers). Species-specific secondary IgG-POD antibodies (Boehringer Mannheim, Indianapolis, IN) were used at 1:250 in conjunction with the Vectastain Elite ABC kit (Vector Laboratories) and peroxidase substrate DAB (Sigma, St Louis, MO) to reveal antigen-antibody complexes.

Semi-quantitative reverse transcriptase polymerase chain reaction (sqRT-PCR)
Yolk sacs from E8.5 embryos were dissected and pooled according to genotype, then RNA was extracted using TRIzol Reagent (Invitrogen) and purified as specified by the manufacturer. RNA (1 µg) was reverse transcribed with random primers and Superscript Reverse Transcriptase II (Invitrogen) at 42°C for 45 minutes and 72°C for 15 minutes. One-twentieth of the RT reaction was PCR amplified using Taq polymerase (Invitrogen) and specific primers as follows: Flik1 (forward, 5′-gccagaaggggaagctctg-3′; reverse, 5′-tttgtatcgtgtgctggtgtg-3′), VE-cad (forward, 5′-gtgccagccgtaaactcaaga-3′; reverse, 5′-accacgccctctcattagctg-3′), Tie2 (forward, 5′-atgacgctctggccaatga-3′; reverse 5′-ctttgacccgcttctgtaac-3′), Ang1 (forward, 5′-cagtcgctgtaactttgatc-3′; reverse, 5′-tgtgcctgctggtgtgtg-3′), Cx40 (forward, 5′-atgagctgctgctgctgctg-3′; reverse, 5′-cacaagatgccagctcacc-3′), HPRT (forward, 5′-cgggctgtaaagcacgtc-3′; reverse, 5′-cacaagatgccagctcacc-3′). Reactions for each primer set were run at their optimized annealing temperature, within their determined linear range and with no RT controls. HPRT was used as
an internal control for semi-quantitative comparison. Each RT-PCR experiment was performed with 4-5 separate RNA collections.

Quantification of cell proliferation and apoptosis in situ

Proliferation
Embryos and yolk sacs (E8.25-9.5) were fixed for 2-3 hours in 4% paraformaldehyde in PBS at 4°C. Endogenous peroxidase activity was quenched via incubation with 3% H2O2 in PBS for 30 minutes, followed by washing in PBS containing 0.2% BSA and 0.1% Triton. Tissues were incubated with anti-phosphohistone 3 antibodies (1:200; Upstate, Lake Placid, NY) overnight at 4°C, then with peroxidase-linked anti-rabbit secondary antibodies (1:250); antigen-antibody complexes were revealed using DAB substrate. Nuclei within tissues were revealed via mounting slides with Vectashield containing DAPI (Vector). The numbers of phosphohistone 3-positive and DAPI-labeled nuclei in each of three high-power fields was counted in five yolk sac samples from wild-type and Raldh2−/− embryos; data represent the mean±s.d. for each group. Immunostaining with anti-phosphohistone 3 antibodies was similarly performed on frozen sections of wild-type and Raldh2−/− yolk sac tissues; sections were co-stained with anti-VE-cadherin and DAPI, as described above.

Apoptosis
Detection of apoptotic cells was performed on 10 μm frozen sections using the DeadEnd colorimetric TUNEL assay system (Promega, Madison, WI). Briefly, slides were fixed in 4% paraformaldehyde for 15 minutes, washed in PBS and permeabilized with 20 μg/ml Proteinase K solution for 10 minutes. Sections were then washed in PBS, repeat fixed in 4% paraformaldehyde, washed in PBS and equilibrated for 10 minutes in equilibration buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 0.2 mM DTT, 2.5 mM cobalt chloride, 0.25 mg/ml BSA). In situ 3’OH ends of DNA fragments were labeled with biotinylated nucleotide mix (250 μM biotinylated nucleotides, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA) by terminal deoxynucleotidyl transferase (25 U) for 1 hour at 37°C. Sections were then sequentially washed in 2xSSC and PBS, and blocked with 0.3% H2O2 for 3 minutes. Biotinylated DNA was detected by incubating with Streptavidin HRP (1:500 in PBS) for 30 minutes and addition of DAB substrate.

Retinoic acid rescue of Raldh2−/− mutants
To determine to what extent vascular defects observed in Raldh2−/− embryos and yolk sacs could be rescued, and to identify the crucial period(s) during vascular development when RA is needed, maternal diet was supplemented with RA during defined treatment periods that correlated with specific stages of vessel assembly (E7.5-8.5; E7.5-9.5; E7.5-12.5). To prepare RA-supplemented diet, all trans RA (Sigma) from a 5 mg/ml ethanol stock suspension was diluted in 50 ml of water and mixed with 50 g of powdered chow to a final concentration of 100 μg/g food, which was partially covered with aluminum foil and renewed each day for up to 5 days. E12.5 embryos were analyzed histologically.

Endothelial cell proliferation assay
Endothelial cells were isolated from adult bovine aortas, as described (Gimbrone, 1976), and grown in Dulbecco’s Modified Essential Medium (DMEM) containing 10% calf serum (CS), penicillin, streptomycin and glucose. For proliferation assays, endothelial cells were trypsinized, counted using a Coulter Counter and plated in 24-well culture dishes at 10,000 cells per well in 0.5 ml DMEM/2% CS. Cells were incubated at 37°C for up to 3 days in the presence of 0-1 μM all-trans retinoic acid. At each time point, triplicate samples were trypsinized and counted. Data represent the mean±s.d. of total endothelial cell number in each experimental group from at least three experiments.

Cell cycle analysis
Endothelial cells were plated and treated with RA as described above for cell proliferation assay; however, after trypsinization, cells were fixed in 100% methanol, then incubated with 10 μg/ml propidium iodide (Calbiochem, San Diego, CA). The cell samples were then filtered through 35 μm nylon mesh and DNA analyzed within an hour on a flow cytometer (Beckon Dickinson FACScan). FACS analysis figures are shown for one representative experiment; data represent averages from at least three experiments.

Western blot analysis
Total protein was isolated from endothelial cells that were cultured in the presence of 0-1 μM retinoic acid for up to 3 days, as previously described (Hirschi et al., 1996), and electrophoresed in 7 to 15% SDS-polyacrylamide gels (10 μg protein per lane). After transfer to a 0.2 μm PVDF membrane (Millipore, Bedford, MA), the membranes were blocked in 5% nonfat dry milk (in PBS with 0.1% Tween20) for 1 hour, then incubated for 1 hour with primary antibodies. Antibodies included mouse monoclonal anti-p21 (Lab Vision, Fremont, CA), diluted 1:500 in blocking solution; mouse anti-p27 (1:750; BD Transduction, San Diego, CA); anti-RB (1:500; Lab Vision); anti-cyclin D1 (1:1000; Rockland); anti-cyclin D2 (1:1000; Lab Vision); anti-Cdk2 (1:1000; Rockland); and anti-Cdk4 (1:750; Rockland); and anti-cytoskeletal actin (1:1000; Santa Cruz). After primary antibody incubation, membranes were washed three times in 0.1% Tween20 in PBS for 20 minutes, then exposed to species-specific secondary antibodies conjugated to horseradish peroxidase (1:10,000; Amersham Life Sciences, Arlington Heights, IL). Antigen-antibody complexes were revealed using an enhanced chemiluminescence system (ECL-Plus kit; Amersham); autoradiographs were quantified using a digital imaging system (Alpha Innotech, CA).

Immunoprecipitation and detection of protein-protein complexes
Total protein was isolated from endothelial cells that were cultured in the presence of 0-1 μM all-trans retinoic acid for up to three days, as described (Hirschi et al., 1996). Cell lysates were pre-cleared using protein A/G agarose, and incubated with primary antibodies overnight at 4°C. The following primary antibodies were used to immunoprecipitate proteins from cell lysates: anti-p21, 1:1000; anti-p27, 1:750; anti-cyclin D1, 1:1000; anti-cyclin D2, 1:1000; anti-Cdk2, 1:1000; anti-Cdk4, 1:1750. Immunoprecipitated protein was pelletted, rinsed, resuspended in gel loading buffer, then boiled and pelletted again. The supernatant was subjected to western analysis, as described above.

Results
RA signaling is crucial for embryonic cardiovascular development, yet its precise cellular role in mammalian blood vessel formation has not been defined. RA-deficient embryos, produced by the targeted deletion of Raldh2 (Niederreither et al., 1999), were used to determine how RA regulates this process. Given the defects in cardiac morphogenesis exhibited by these mutants (Niederreither et al., 2001), it was unclear whether RA-deficiency induced primary defects in the vasculature, or if the observed abnormalities resulted from cardiac malfunction. Thus, we evaluated vascular cell differentiation, maturation, proliferation and apoptosis, as well as blood vessel assembly, in Raldh2−/− embryos and yolk sacs and wild-type littermates prior to, and following, the onset of cardiac function and blood circulation.

Vascular plexus formation and remodeling
Embryos and yolk sacs were dissected at E8.25-8.5 (3- to 6-somite stage), prior to the onset of cardiac function and
systemic blood circulation, photographed, then genotyped and processed for further analyses. Upon morphological examination, it was apparent that a vascular plexus had formed in both wild-type and Raldh2–/– yolk sac tissues (Fig. 1A-D). Hematoxylin and Eosin staining of 5 µm paraffin wax-embedded sections of yolk sac tissue suggested that although a vascular plexus formed in both wild type and Raldh2–/– mutants (Fig. 1C,D), the vessels within Raldh2–/– plexi were dilated (Fig. 1D).

The effects of RA deficiency on vascular remodeling and vessel assembly were further evaluated at E9.5. Upon morphological examination of wild-type yolk sacs, it was apparent that the initial vascular plexus that formed was appropriately remodeled into a well-defined, branched network that circulated blood throughout the embryo (Fig. 1E). By contrast, the yolk sacs of Raldh2–/– mutants were not remodeled and lacked large vitelline vessels (Fig. 1F).

To determine whether the endothelial tubes formed in wild-type and Raldh2–/– tissues acquired a vessel wall composed of mural cells (smooth muscle cells or pericytes), yolk sac tissues isolated at E9.5 were immunostained with antibodies against SM-α-actin, which is among the first proteins to be significantly upregulated in mesenchymal progenitors in response to endothelial cell recruitment and contact (Hungerford et al., 1996; Hirschi et al., 1998). In E9.5 wild-type tissues, a high level of SM-α-actin expression was evident around all large vessels of the yolk sac (Fig. 1G), and associated with a small proportion of vessels in the head region and along the dorsal aorta within the embryo proper (not shown). By contrast, whole-mount staining of Raldh2–/– mutants revealed that the endothelial tubes in yolk sacs (Fig. 1H) and embryos (not shown) were not invested with cells highly expressing SM-α-actin, suggesting lack of endothelium-mediated mural cell recruitment and/or differentiation.

Endothelial cell maturation

As morphological defects were apparent in Raldh2–/– mutants prior to the onset of cardiac function and vessel wall formation, we focused all subsequent cell and molecular analyses on mutants at E8.25–8.5. In so doing, we were able to define the direct effects of RA on endothelial cell behavior and early vessel formation and avoid potential confounding effects of RA deficiency that may occur at later stages of development, such as disrupted hemodynamic forces and systemic oxygen/nutrient distribution.

We performed whole-mount immunostaining of E8.25–8.5 embryos and yolk sacs with antibodies against PECAM1, an early marker of endothelial cells. We found that in wild-type yolk sacs, endothelial tubes were similar in diameter, evenly distributed, and exhibited some ‘pruning’ into smaller vessel structures (Fig. 2A, arrows). By contrast, endothelial tubes within Raldh2–/– yolk sacs were dilated, with little evidence of appropriate remodeling (Fig. 2B, arrows). Within wild-type embryos, vascular plexus formation was evident within the head (Fig. 2C) where distinct margins of vascularization were notable (arrows). By contrast, the vascular plexi within the heads of Raldh2–/– embryos were diffusely distributed (Fig. 2D). Vessels within the somitic regions of Raldh2–/– mutants were also dilated and inappropriately patterned (Fig. 2F, arrows) relative to wild type (Fig. 2E).

To assess the degree of endothelial cell differentiation and maturation in Raldh2–/– mutants compared with wild type, we performed sqRT-PCR analyses of total RNA isolated from the yolk sacs of mutant, heterozygote and wild-type littermates. We found that RA deficiency had no effect on early endothelial cell differentiation, as evidenced by wild-type levels of Flk1, VE-cadherin and Tie2 mRNA in Raldh2–/– mutants compared with wild-type littermates (Fig. 2G). The expression of Ang1 in Raldh2–/– mutants was also similar to wild type, suggesting no effect of RA deficiency on mural cell precursors. There was,
Control of vascular cell proliferation and apoptosis

The dilated and immature vessel structures in Raldh2−/− yolk sacs and embryos could result from multiple endothelial cell dysfunctions. Given the proposed role of retinoids in growth control and programmed cell death in other systems, we examined whether vessel defects in the Raldh2−/− mutants resulted from lack of endothelial cell growth control or suppressed apoptosis during vascular development. To obtain a measure of cell proliferation in vivo, we immunostained wild-type and Raldh2−/− yolk sac and embryonic tissue (five tissues in each group) with antibodies against phosphohistone 3, which is detectable only in cells undergoing mitosis. Representative stained tissues are shown in Fig. 3A,B; there was consistently more phosphohistone 3 staining in Raldh2−/− tissues.

The numbers of phosphohistone 3-positive and DAPI-stained nuclei per high-power field were counted in three different regions of each of five tissues to determine the proportion of mitotically active cells in wild-type and mutant tissues. The mitotic index (number of phosphohistone 3-positive/total number of nuclei) for each tissue was calculated as the mean±s.d. of the 15 random fields counted for each experimental tissue. The mitotic index was significantly higher in Raldh2−/− tissues compared with wild type at E8.5 (6.3±1.5% versus 2.7±1.0%, respectively; Fig. 3A,B), as well as E9.5 (5.4±0.8% versus 2.4±0.7%, respectively; not shown).

To determine which cell types within Raldh2−/− tissues exhibited increased mitotic index, cross-sections of E8.5 yolk sacs were immunostained for phosphohistone 3 and co-stained with DAPI; mitotic index was calculated as described above. We found no difference in the mitotic index of cells within the visceral endoderm layer of wild-type and Raldh2−/− yolk sacs (Fig. 3C); however, there was a significant increase in mitosis in the mesodermal layer of Raldh2−/− yolk sacs compared with wild type. Co-immunostaining for phosphohistone 3 and endothelial cell marker VE-cadherin demonstrated that the mitotic cells in the Raldh2−/− yolk sacs were predominantly vascular endothelial cells (Fig. 3E). There was no difference in the level of apoptosis among endothelial cells in the wild-type and Raldh2−/− yolk sacs (data not shown).

We examined the expression of cell cycle inhibitors via whole-mount and section immunostaining of wild-type and Raldh2−/− yolk sacs. We found significantly reduced levels of p21 (Fig. 4B) and p27 (not shown) in Raldh2−/− tissues, compared with wild type (Fig. 4A). Section staining revealed that p21 expression in the wild-type yolk sac was predominantly in endothelial cells of blood vessels in the mesodermal layer of the yolk sac (Fig. 4C, arrows); p21 protein was essentially absent in Raldh2−/− yolk sacs (Fig. 4D).

RA regulation of endothelial cell cycle progression

Immunohistochemical analyses have suggested that, during normal vascular development, RA induces the expression of p21 and p27 in endothelial cells to control cell cycle progression, and in the absence of RA, endothelial cell growth is not controlled, which disrupts the processes of blood vessel assembly and remodeling. To determine more directly whether RA regulates the expression of cell cycle associated proteins and controls cell cycle progression in endothelial cells, we studied the effects of RA on their growth and cell cycle regulation in vitro. As biochemical studies require large numbers of cells, we isolated endothelial cells from adult bovine aortas. Importantly, we have recently found that the effects of RA on the growth of adult rat microvascular endothelial cells and E9.5 embryonic mouse tissues are similar to results obtained in adult bovine endothelial cell experiments elaborated below.
Endothelial cells were cultured for up to 72 hours in the presence of 0-1 µM all-trans RA, and the expression of cell cycle associated proteins was examined. Via western blot analyses, we determined that 1 µM RA induced the expression of p21 (Fig. 4E) and p27 (not shown), members of the Cip/Waf family of Cdk inhibitors, within 8 hours, but had no affect on the expression of members of the Ink4 family of Cdk inhibitors (p15, p18, p19) or p53 (data not shown). Retinoid treatment also had no effect on the expression of cyclins A, B, D3 or E, or on the expression of Cdc2, or Cdk2, 5 or 6 (data not shown). Surprisingly, retinoids upregulated the expression of cyclin D1 and D2 proteins and Cdk4 (not shown), which are associated with progression of cell cycle from G1 to S phase. The upregulation of cyclin D proteins, in conjunction with the upregulation of p21 and cell cycle arrest of fibroblasts has been previously reported (Sinibaldi et al., 2000), but the biological significance is not clear.

To determine whether RA directly regulates endothelial cell proliferation, we cultured endothelial cells for 3-5 days in the presence of 0-1 µM all-trans RA or 0-1 µM synthetic agonists (BioMol) of the RXR or RAR receptor families – methoprene acid (RXR-specific agonist) and TTNPB (RAR-specific agonist). RA significantly suppressed the increase in endothelial cell number seen over time in culture in untreated cells, as did the RAR agonist TTNPB (Fig. 5A); RXR agonist MA had no effect on endothelial cell number. Thus, it appears that RA regulates endothelial cell number in a process specifically mediated via RAR receptors. The effects of RA on endothelial cell number were dose-dependent from 0.1 to 5 µM (data not shown). After cell counting, control and RA-treated endothelial cells were fixed and stained with propidium iodide, then subjected to fluorescence activated cell sorting (FACS) to determine their cell cycle distribution. In response to RA, there was a significant decrease in the proportion of endothelial cells in S phase and an increase in the proportion of cells in G1 phase (Fig. 5B). There was no evidence of RA-induced endothelial cell apoptosis, which corroborates our in vivo findings.

To determine the mechanism by which RA induces cell cycle arrest in endothelial cells, and whether p21 and p27 are directly involved, we performed immunoprecipitation experiments. We were specifically interested in determining whether RA-induced p21 and p27 proteins interfered with complexing
between cyclin D and Cdk4 proteins, which is needed for proper function of Cdk4, phosphorylation of Rb protein, and progression from G1 to S phase of cell cycle (Ekholm and Reed, 2000). Total protein was isolated from control or RA-treated endothelial cells, immunoprecipitated with antibodies against Cdk4, p21 or p27, and subjected to western blot analyses. We found that complex formation between cyclin D1 or D2 and Cdk4 was significantly reduced in RA-treated endothelial cells; whereas, complex formation between RA-induced p21 and Cdk4, as well as cyclins D1 and D2, was greatly enhanced (Fig. 6A). Similar results were obtained when immunoprecipitation was performed using p27 antibodies (data not shown). Reduced complex formation between Cdk4 and cyclin D proteins presumably caused the decreased Cdk activity that yielded lowered levels of phosphorylated Rb protein observed in RA-treated endothelial cells, compared with untreated controls (Fig. 6B).

In summary, these in vitro studies corroborated our in vivo observations. That is, RA appears to control endothelial cell cycle progression from G1 to S phase via the upregulation of Cdk inhibitors. Enhanced levels of p21 and p27 proteins in RA-treated cells competitively inhibit complex formation between Cdk4 and cyclin D proteins that is needed for Cdk activity; thus, suppressing phosphorylation of Rb and transition from G1 to S phase of cell cycle.

Circulating RA rescues Raldh2−/− vascular defects

It was previously determined that the effects of Raldh2 deficiency on the embryo could be partially rescued by providing RA to the embryos via maternal diet and blood circulation (Niederreither et al., 2001; Niederreither et al., 2002). RA administration at a dosage of 100 μg/g food (see Materials and methods) does not interfere with RARE-lacZ reporter transgene activity and safely avoids teratogenic effects in wild-type embryos (Niederreither et al., 2002). We used this system to determine to what extent vascular defects observed in Raldh2−/− embryos and yolk sacs could be rescued and to identify the crucial period(s) during vascular development when RA is needed. We supplemented maternal diet with RA during defined developmental periods that correlated with specific stages of vessel assembly. In this manner, we studied the impact of circulating RA on endothelial tube formation (E7.5-8.5 RA supplementation); vascular remodeling, including initial mural cell recruitment (E7.5-9.5 RA supplementation); and vessel assembly, including mural cell differentiation and vessel wall formation (E7.5-12.5 RA supplementation).

Pregnant Raldh2+/– females were fed powdered chow containing 100 μg/g all-trans RA from E7.5-8.5, E7.5-9.5 or E7.5-12.5; all mice were killed at E12.5. Wild-type embryos from the experimental (Raldh2+/–) litters and E12.5 wild-type embryos from chow-fed animals were used as controls, and there was no difference in the development of these embryos, indicating that RA had no effect on the development of wild-type embryos. Almost all mutants of RA-fed mothers survived
to E12.5, whereas mutant embryos of chow-fed females died at E10.5. The embryos exposed to RA from E7.5-8.5 (during endothelial tube formation) exhibited the lowest degree of rescue of vascular development (Fig. 7, column 2). The yolk sacs of these embryos exhibited formation of some large vessels (arrowheads), but all smaller caliber vessels (arrows) were significantly dilated. Exposure to RA from E7.5-9.5 (third column) induced formation of a larger number of branched vessels (arrowheads), but dilation of small vessels (arrows) was still evident. Exposure to RA from E7.5-12.5 (fourth column), lead to vascularization of mutant yolk sac equivalent to wild type; large (arrowheads) and small (arrows) vessels were similar in caliber and distribution. The mitotic index of yolk sac tissue (bottom row) from ‘rescued’ mutants was evaluated via immunostaining for phosphohistone 3 and significantly elevated for all, compared with wild type, except those exposed to RA at E7.5-12.5.

Discussion
Factors such as bFGF and VEGFA are known to stimulate endothelial lineage commitment and replication needed to initiate vascular development; however, it is unclear how endothelial cell growth is suppressed. Our data herein provide evidence that RA serves this function – to suppress endothelial cell cycle progression, which is needed for vascular plexus remodeling and later stages of vessel assembly. Thus, insights gained from these studies may prove useful for the modulation of neovascularization during the progression of prevalent pathologies and for the control of vessel assembly ex vivo in tissue engineering applications.

We found that in the absence of RA (Raldh2−/− mutants), mesodermal progenitors do differentiate into endothelial cells that form a primitive vascular plexus. However, the blood vessels that constitute the plexi of RA-deficient embryos are immature, abnormally dilated and never become remodeled into a competent circulatory network capable of sustaining embryonic development. We determined that the dilation and inappropriate patterning of blood vessels observed in the absence of RA was associated with lack of endothelial cell cycle control. Uncontrolled endothelial cell growth during early vessel formation in Raldh2−/− mutants disrupted later stages of vessel assembly, as well, including endothelial-induced mural cell recruitment and differentiation.

The fact that circulating RA, provided to the embryo through
maternal diet, restored cell cycle control and rescued the observed vascular defects in Raldh2−/− embryos and yolk sacs indicates that endothelial cells themselves need not produce active forms of RA during development. However, given that active RA would not normally be obtained from maternal circulation, which typically contains the precursor retinol, our studies indicate that functional embryonic Raldh2 is required for normal vascular development. Furthermore, as RA was needed during the entire period of vascular development to suppress endothelial cell growth and restore vascular remodeling, it appears that continuous regulation of the production or degradation (White et al., 1997; White et al., 1996) of this soluble effector is necessary for appropriate vessel formation and function.

Our in vivo and in vitro data collectively suggest that RA signaling suppresses endothelial cell replication via the upregulation of Cdk inhibitors of the Cip/Waf family. Consistent with our findings, previous studies demonstrated that the expression of p21 and p27 is transcriptionally (Liu et al., 1996; Sasaki et al., 2000) and post-transcriptionally (Dimberg et al., 2002) regulated by RA. We found that in endothelial cells in vitro, the inhibitors p21 and p27 competitively bind to Cdk4 and cyclin D proteins and, thus, prevent their interactions, which are needed for Cdk4-dependent progression from G1 to S phase of cell cycle (Ekholm and Reed, 2000). This mechanism of RA control of proliferation has been previously demonstrated for lymphocytes (Naderi and Blomhoff, 1999) and myeloid cells (Dimberg et al., 2002), as well as tumor cells (Hsu et al., 2000; Suzui et al., 2002; Zhang et al., 2001).

Although the timing of RA-induced expression of p21 and p27 in endothelial cells within 8 hours in vitro is consistent with transcriptional or post-transcriptional regulation, it is possible that RA signaling in vivo functions in conjunction with other signaling pathways to modulate endothelial cell maturation and growth control. For example, TGFβ signaling is associated with the regulation of endothelial cell extracellular matrix production (Goumans et al., 1999), which may contribute to growth control. In addition to upregulation of p21 at 8 hours, we also observed an increase at 72 hours; therefore, it is possible that RA induces other signals such as the TGFβ, which contribute to endothelial cell cycle control via matrix production and cell-matrix mediated interactions. A signaling hierarchy among these factors, and their potential contribution to endothelial cell cycle control in vivo, are currently under investigation. However, the fact that maternaly derived RA is sufficient to rescue the endothelial cell proliferation defects in Raldh2−/− mutants indicates that if other signaling pathways are involved in the control of endothelial cell cycle progression, they are downstream of RA signaling.

Endothelial cell proliferation has also been proposed to be passively controlled via the VEGFA receptor Flt1. Similar to Raldh2−/− mutants, Flt1-deficient embryos exhibit abnormally high levels of endothelial cell replication, associated with disrupted vascular remodeling and vessel assembly (Fong et al., 1995). Flt1 is proposed to modulate endothelial cell proliferation by limiting the local availability of VEGFA and, thereby, preventing the stimulation of pathways that trigger replication (Kearney et al., 2002). Consistent with this idea, we found that Flt1 mRNA expression was downregulated in the Raldh2−/− embryos; however, VEGFA levels were concomitantly reduced (B.L.B., L.L., Pascal Dolle and K.K.H., unpublished). Therefore, it is unlikely that decreased Flt1 receptor availability in Raldh2−/− mutants would lead to enhanced endothelial cell replication induced by excessive VEGFA signaling.

Given that circulating RA suppresses endothelial cell cycle progression during vessel assembly, it may be possible to use RA (provided intravenously or orally) to treat pathologies in which uncontrolled neovascularization is a primary defect, such as in angiogenesis-dependent tumorgenesis or retinopathy. In support of this idea, it was previously suggested that suppression of endothelial cell proliferation contributes to RA-induced growth inhibition of squamous cell carcinoma in vivo (Lingen et al., 1996).

In summary, we have demonstrated that RA plays a crucial role in the control of mammalian vascular development. The primary role of RA appears to be the suppression of endothelial cell growth during vascular remodeling and patterning; no other soluble factor, to date, has been shown to control this process in vivo. Requirement of a circulating factor to inhibit endothelial cell growth may explain why the establishment of fully formed, quiescent blood vessels is coincident with and dependent upon the onset of blood circulation. Further elucidating the mechanism(s) by which RA signaling controls endothelial cell cycle control and vascular remodeling during normal development should provide the necessary insights into the control of aberrant neovascularization associated with prevalent pathologies.

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References


