Angiogenesis gene downregulation in placentae of ARNT-deficient embryos.

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Introduction

The aryl hydrocarbon nuclear translocator protein (ARNT) is a member of the basic helix-loop-helix/ Per-ARNT-Sim (bHLH/PAS) family of transcription factors. ARNT interacts with several other members of the bHLH/PAS family, including the hypoxia inducible factor HIF-1", SIM-1 (single-minded 1) and SIM-2, and the aryl hydrocarbon receptor (AhR). The AhR binds ligand and after dimerizing with ARNT regulates transcription of a number of target genes, including *CYP1A1*. HIF-1" dimerizes with ARNT and is involved in mediating cellular responses to hypoxia, including increased expression of vascular endothelial growth factor (VEGF) and endothelial PAS domain protein 1 (EPAS1/HLF).

Vasculogenesis and angiogenesis require a complex pathway of gene expression that includes VEGF and several receptors that bind VEGF. VEGF exists in several isoforms and knockout of genes for VEGF or its receptors is embryolethal due to failure of vasculogenesis in yolk sac, placenta, and/or the embryo. The differentiation of endothelial cells and their subsequent organization to form blood vessels depends on expression of VEGF, VEGF receptors and binding of VEGF to those receptors. Transgenic knockout of the VEGF gene is lethal in heterozygotes and the +/- transgenic embryos exhibit significant defects in the vasculature of the placenta. The VEGF tyrosine kinase receptor Flk-1 (VEGF-R2) is expressed in the labyrinthine layer of the placenta. VEGF-R2 binds both VEGF and placental growth factor, and is essential for the organization of embryonic vasculature. EPAS1 (HLF) also interacts with ARNT and is thought to be a regulator of vascularization. EPAS1 can activate expression of the endothelial-specific tyrosine kinase receptor Tie-2 (Tek). The *tie*-2 gene is expressed in the early embryonic vascular system, in maternal decidual vascular endothelial cells, and in extraembryonic mesoderm cells of the amnion. A *tie*-2 knockout dies by GD 10.5 and has been shown to affect angiogenic processes of endothelial cells.

ARNT is expressed during development in placental and embryonic tissues with specific temporal and spatial expression patterns. Transgenic knockout of the *Arnt* gene is lethal by gestation day (GD10) and this lethality is attributed to failure of the embryonic component of the placenta to vascularize and form the labyrinth. The involvement of ARNT in regulation of VEGF and the placental deficiencies in *Arnt* -null transgenic mice raises the possibility that both VEGF and ARNT are involved in normal placental formation. The VEGF pathway is required for embryonic survival and includes several receptors (VEGFR1, VEGFR2, Tie2) and ligands (VEGF, Ang1, Ang2, neuropillin). This study examines the VEGF pathway in GD9.5 embryos of wild type (+/+), heterozygous (+/-), or knockout (-/-) ARNT genotype.

Materials and Methods

Arnt-deficient mice were thus maintained as a heterozygous (+/-) colony. The *Arnt*-deficient transgenics were derived as described in Kozak et al., 1997. The animals were housed under controlled conditions of temperature (72±2EF), humidity (40-60%), and lighting (12/12-hr light/dark cycle), and provided food and water *ad libitum*.

Tissue collection and genotypic analysis: Heterozygous pairs were mated overnight and females were weighed and examined for presence of a copulatory plug the next morning which was then designated gestation day (GD) 0.5. Pregnant females were killed by carbon dioxide inhalation on GD9.5, the uterus was removed and placed in PBS for removal of the conceptuses. For each conceptus, a small incision was made in the extraembryonic membranes, the embryonic head gently coaxed outside the membranes and the embryo was decapitated using Dumont's #5 forceps. (For some embryos a tissue sample was collected from the posterior rather than anterior region.) The embryonic tissue was immediately frozen to be used later for genotypic analysis by PCR. The conceptus was then fixed in 3% paraformaldehyde on ice for 30 min, dehydrated by ethanolic series, and embedded in paraffin. Sections were prepared for immunohistochemical analysis such that each slide had sections from +/+, +/-, and -/- embryos. The genotypes were determined by PCR analysis of genomic DNA prepared using the Gentra™ DNA Purification System (Generation Systems, Inc.), in which a small piece of the embryonic tissue (usually half of the GD9.5 head) was absorbed onto the Gentra DNA disk. The disk+tissue was allowed to stand at room temperature for 1 hour, then washed with the buffers provided to prepare genomic DNA. The PCR reaction was run using primers corresponding to the bHLH domain (*Arnt* forward, 5'-CATAGTGAAATAGAACGGCGGCG-3'; *Arnt* reverse, 5'-AGGACTTCATGTGAGAAACGGC-3') and the *neo* gene reverse primer, 5'-TGGCGGCGA ATGGGCTGACC-3').

Immunohistochemistry: Antibodies to VEGF, VEGFR1, VEGFR2, Tie2, Ang1, Ang2, neuropillin, ARNT, and platelet endothelial cell adhesion factor (PECAM) were incubated with sections and localized by secondary antibody-avidin-biotin-peroxidase complexes. Antibody specificity was tested by preincubation with specific peptides. Differentiated endothelial cells were localized using a purified anti-mouse PECAM antibody, CD31 (Phar-Mingen; San Diego, CA). Antibody was diluted 1:1000, incubated over the sections overnight, and detected with biotin conjugated anti-rat AffiniPure antibody (Jackson Immunoresearch Laboratories, West Grove, PA) and the Vector ABC Kit for peroxidase (Vector Labs, Burlingame, CA). VEGF was localized using a polyclonal antibody prepared against recombinant mouse VEGF, expressed in Sf-21 insect cells using a baculovirus expression system (AF-493-NA, peptide 493-M, R&D Systems, Inc, Minneapolis, MN). Antibodies recognizing VEGFR1 (Flt1), VEGFR2 (Flk1), Tie2, Ang1, Ang2 and Neuropillin were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, (SC-316; SC-315G; SC-324; SC-6320; SC-7017; SC-7217, respectively). Primary antibodies were incubated with sections overnight and detected using appropriate biotinylated secondary antibody and the Vector ABC Kit with diaminobenzidine substrate for peroxidase. In addition, each antibody was incubated in solution overnight with blocking peptide (specific for each antibody; Santa Cruz Biotechnology, Santa Cruz, CA). Incubation with the specific immunogen

used to prepare the antibody was expected to bind to the antibody and inhibit specific immunostaining of the tissue sections.

Results and Discussion

ARNT is known to heterodimerize and regulate levels of VEGF through the HIF1" pathway. The *Arnt*-null conceptuses expressed VEGF similarly to the wild type embryos. Thus, the dimerization of ARNT with HIF1" does not appear to be required for expression of VEGF in the placenta. However, it may be that in situations leading to hypoxia for the conceptus, the HIF-1" pathway could provide an important adaptive or compensatory pathway.

The poor development of the placental labyrinth of *Arnt*-deficient conceptuses correlated with reduction in PECAM-expressing endothelial cells lining the cavities and decreased binding of VEGF. The deficient binding of VEGF in *Arnt*-deficient placentas could be due to reduced expression of a specific VEGF receptor and/or ability of receptors to bind the required VEGF isoform for that particular tissue to develop. For example, VEGF-R2 (Flk) mRNA is expressed in the labyrinthine layer of the placenta. In vasculogenesis, the binding of VEGF to VEGF-R2 regulates recruitment of progenitor cells, proliferation and differentiation of endothelial cells. If the *Arnt*-deficient placenta did not express VEGF-R2 or the receptor failed to bind VEGF, then the expected outcome would be fewer differentiated endothelial cells. This appears to be the case for the -/- placental labyrinth on GD9.5. All genotypes expressed abundant VEGF in trophoblastic giant cells. However, -/- conceptuses had less VEGFR2 in placental labyrinthine and giant cells. Tie2 expression also appeared to be decreased in trophoblasts and giant cells surrounding the yolk sac. Ang1 was only detected at very low levels in the placenta but appeared to depend on genotype with +/+expression highest (+/+>+/->-/-). The effects on VEGF pathway genes are implicated in failure of placental development in the ARNT-deficient embryo.

References

Kozak K.R., Abbott B. and Hankinson O. (1997). *Dev. Biol.* **191,** 297-305.

(This abstract does not reflect US EPA policy).