Dioxin Receptor Deficiency Impairs Angiogenesis by a Mechanism Involving VEGF-A Depletion in the Endothelium and Transforming Growth Factor-β Overexpression in the Stroma*

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Angiogenesis has key roles in development and in the progression of human diseases such as cancer. Consequently, identifying the novel markers and regulators of angiogenesis is a critical task. The dioxin receptor (AhR) contributes to vascular homeostasis and to the endothelial response to toxins, although the mechanisms involved are largely uncharacterized. Here, we show that AhR-null mice (AhR−/−) have impaired angiogenesis in vivo that compromises tumor xenograft growth. Aortic rings emigration experiments and RNA interference indicated that AhR−/− endothelial cells failed to branch and to form tube-like structures. Such a phenotype was found to be vascular endothelial growth factor (VEGF)-dependent, as AhR+/− aortic endothelial cells (MAECs) secreted lower amounts of active VEGF-A and their treatment with VEGF-A rescued angiogenesis in culture and in vivo. Further, the addition of anti-VEGF antibody to AhR+/− MAECs reduced angiogenesis. Treatment under hypoxic conditions with 2-methoxyestradiol suggested that HIF-1α modulates endothelial VEGF expression in an AhR-dependent manner. Importantly, AhR-null stromal myofibroblasts produced increased transforming growth factor-β (TGFβ) activity, which inhibited angiogenesis in human endothelial cells (HMECs) and AhR−/− mice, whereas the co-culture of HMECs with AhR−/− myofibroblasts or with their conditioned medium inhibited branching, which was restored by an anti-TGFβ antibody. Moreover, VEGF and TGFβ activities cooperated in modulating angiogenesis, as the addition of TGFβ to AhR−/− MAECs further reduced their low basal VEGF-A activity. Thus, AhR modulates angiogenesis through a mechanism requiring VEGF activation in the endothelium and TGFβ inactivation in the stroma. These data highlight the role of AhR in cardiovascular homeostasis and suggest that this receptor can be a novel regulator of angiogenesis during tumor development.

The formation of new blood vessels is an important task that takes place in healthy tissues but also during tumor development. Among the several mechanisms of neovascularization already known, angiogenesis is particularly relevant for tumor vasculature and consists in the formation of new blood vessels by sprouting from preexisting ones (1, 2). Angiogenic sprouting and branching involve specific populations of endothelial cells that guide tube formation at the tip and respond to vascular endothelial growth factor (VEGF) as well as stalk cells that proliferate to form the vascular structure (3, 4). The control of endothelial cell branching is regulated by both positive and negative factors that ultimately determine angiogenesis (5, 6). A large body of evidence strongly suggests that the endothelium-specific factor VEGF has a prominent role in angiogenesis and is indispensable for vascular development (1, 7–10). Thus, blocking VEGF-dependent signaling by inactivation of the Vegfr-3 gene severely impairs vascular network development in the mouse (4), whereas neutralizing antibodies against the VEGFR-3 receptor reduces vascular density, decreases sprouting and branching (4), and inhibits tumor growth by blocking angiogenesis (11). Importantly, antibody-based strategies have been recently transferred to the clinic in order to inhibit metastatic colorectal cancer angiogenesis by blocking VEGF-dependent signaling through patient treatment with the humanized antibody bevacizumab (Avastin) (12).

Angiogenesis, on the other hand, not only depends on the characteristics of the endothelial cells but also on the interactions that they establish with other stromal cells, in particular with fibroblasts and pericytes (13). Despite the fact that fibroblasts have different properties depending on their tissue of origin, the analysis of α-smooth muscle actin-expressing myofibroblasts isolated from a panel of breast carcinomas revealed common patterns of gene expression (14), which supports their conserved role in the synthesis and maintenance of extracellular matrix (ECM) components. Transforming growth factor-β (TGFβ) is a cytokine secreted and activated in the ECM by...
AhR Alters TGFβ and VEGF in Angiogenesis

mesenchymal cells (e.g. fibroblasts) that exerts a relevant role in proliferation, differentiation, apoptosis, and migration (15). Interestingly, TGFβ is also a major molecule in the regulation of endothelial cell behavior (16) and vascular development by functional interaction with VEGF (17). Indeed, low extracellular TGFβ levels promote endothelial cell proliferation and migration and new blood vessel formation, whereas high TGFβ concentrations induce differentiation of endothelial cells, inhibition of tube formation, and impaired invasion in gels (18–21). In addition, previous work has shown that TGFβ is as potent as VEGF in inducing angiogenesis and that VEGF could be a target for TGFβ (22). Therefore, it is reasonable to assume that altered VEGF and TGFβ activities could induce defects in angiogenesis.

The aryl hydrocarbon (dioxin) receptor (AhR) is a well known transcription factor with increasing importance in cellular physiology and tumor development. Remarkably, AhR has a relevant role in vascular development and homeostasis (23, 24) and in TGFβ activation (25). AhR-null mice fail to resolve the embryonic structure known as portosystemic shunting and thus exhibit a patent ductus venous in the adult liver (23, 24). AhR expression also affects the cardiovascular system, as AhR−/− mice have a significant heart hypertrophy (26, 27), which is probably associated with the hypertension and increased endothelin-1 expression reported in this animal model (28). Previous studies using AhR-null mice have shown that this receptor regulates TGFβ activation in mouse embryonic primary fibroblasts (29) and TGFβ levels in the liver (30). TGFβ overactivation results in decreased proliferation (29, 31) and migration (32, 33) in AhR−/− fibroblasts and co-localizes with portal fibrosis in AhR−/− mouse liver (34). Thus, AhR could be a common signaling intermediate in the regulation of vascular homeostasis by growth factors and cytokines such as TGFβ.

In this study, we sought to analyze the contribution of AhR expression to endothelial cell function as related to vessel formation and angiogenesis. Using mouse and human endothelial cells, tumor xenografts, aortic ring explants, in vivo vessel recruitment to Matrigel, and co-cultures of endothelial and fibroblastic cells, we found that lack of AhR expression significantly impaired formation of tubular structures from aortic explants and angiogenesis and tumor development in vivo. Such a phenotype was a consequence of reduced VEGF activity in AhR−/− endothelial cells and of increased TGFβ activity in AhR-null mesenchymal myofibroblasts. We suggest that AhR is a regulator of angiogenesis and that its down-modulation could represent a potential novel strategy to inhibit tumor growth.

EXPERIMENTAL PROCEDURES

Reagents and Cell Lines—Matrigel and FITC-labeled anti-CD102 were purchased from BD Biosciences and mouse recombinant VEGF-A164 from Calbiochem. Heparin, endothelial cell growth factor supplement, recombinant TGFβ, neutralizing anti-VEGF-A and anti-β-actin antibodies, CoCl2, and 2-methoxyestradiol (2-Me E2) were from Sigma-Aldrich. Antibodies against AhR and CD31 were purchased from Biomol and Abcam, respectively. A neutralizing anti-TGFβ antibody that recognizes all three TGFβ isoforms was obtained from R&D Systems (1D11 clone). Mouse melanoma B16F10 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen), and human endothelial HMEC-1 cells used MCDB-131 medium (Invitrogen). Media were supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

The concentrations of VEGF-A used (100 ng/ml in aortic ring assays or 300 ng/ml in Matrigel plugs in vivo) were shown to efficiently modulate VEGF activity (35, 36). The anti-VEGF neutralizing antibody was used at 200 ng/ml based on the LD50 provided by the manufacturer in human umbilical vein endothelial cells (50–150 ng/ml). Recombinant TGFβ was employed at 10 ng/ml taking into account our previous studies on cytokine activation in the ECM (37) and keratinocyte migration (38). The neutralizing anti-TGFβ antibody was used at 1 μg/ml considering its LD50 (in-house determined to equal 60 ng/ml) (29) and its effect on keratinocyte migration (38).

Mice—AhR-null mice were produced by homologous recombination in embryonic stem cells as described (39). Mice were used at 10–12 weeks of age to obtain endothelial cells or aortic rings or at 30 weeks for in vivo Matrigel plug assays. The experiments involving animals were performed in compliance with the guidelines established by the Animal Care and Use Committee of the University of Extremadura.

Aortic Ring Assays and MAEC Isolation—Aortic Ring Assays and MAEC isolation were done as described previously (40) with some modifications. Briefly, the thoracic aorta was removed and sectioned in 1-mm aortic rings, which were embedded in Matrigel. After solidification, Dulbecco’s modified Eagle’s medium-F12 medium (Invitrogen) containing 20% fetal bovine serum, 200 units/ml penicillin, 200 μg/ml streptomycin, and 250 ng/ml endothelial cell growth supplement was added. The length of the spraying vessels was measured at different times using light microscopy or 4,6-diamidino-2-phenylindole staining. MAECs were isolated at day 12 by solubilizing Matrigel-embedded aortic rings outgrowths in Matrisperse (BD Biosciences) followed by seeding in the same medium. MAEC cultures were assessed for purity by quantifying CD102-positive cells by flow cytometry as described previously (40). In some experiments, VEGF-A was added to the aortic rings at 100 ng/ml.

Induction of Hypoxia by Cobalt Chloride Treatment—To analyze the effects of hypoxia on VEGF expression, AhR+/+ and AhR−/− MAECs were cultured in complete medium and then treated with solvent (DMSO) or with 100 μM CoCl2 for 16 or 24 h. RNA was purified and used to analyze VEGF mRNA levels as indicated below. This concentration of CoCl2 has been shown to efficiently mimic hypoxia in cell culture (41). In these experiments, VEGF mRNA levels were normalized by β-actin because hypoxia can alter Gapdh expression (see primers used in Table 1). The contribution of hypoxia inducible factor-1α (HIF-1α) in maintaining VEGF levels was determined by adding 5 μM 2-Me E2 to CoCl2-treated MAECs or to AhR small interfering RNA (siRNA)-transfected HMEC-1 cells. This concentration of 2-Me E2 was shown to induce degradation of HIF-1α in cultured cells (42, 43).

Tumor Xenografts—Aliquots of 5 × 10⁵ B16F10 cells (in 100 μl of phosphate-buffered saline) were injected subcutaneously...
AhR Alters TGFβ and VEGF in Angiogenesis

TABLE 1
Primer sequences used to analyze the expression of angiogenesis-related genes by real-time RT-PCR

| Gene name | Primer sequences |
|-----------|-----------------|
| Hif-1a    | Forward: TGAAGCTTCAACTCATGAGATAT | Reverse: TAAGGCACTATGTTGGATTC |
| VEGF-A    | Forward: ACACTTCGAGCCGCCCTGTGGC | Reverse: AAATGCGGAACTTCAAAGCACG |
| VEGF-B    | Forward: GGCTGAGAACAGGGCAGGACA | Reverse: GGCTGAGAACAGGGCAGGACA |
| Plg       | Forward: TAAACAAGCCACTCATGAGAGA | Reverse: AGGGGAACTGCTGGCTTAAATA |
| VEGF-1    | Forward: GAGGAGGATGAGGAGGAGGAG | Reverse: GGTGATCAGCTCCAGGTTTGACTT |
| VEGF-2    | Forward: GCCGTCCGTGGCTCTCTACAT | Reverse: CAAAGCTCGCTCTCCATGAT |
| VEGF-A isoforms | Common forward: GCCGACCATAGGAATGGACCC |
| TGFβ-1    | Forward: TGGCCCTGCAGAAATTTTAA | Reverse: AGCCGGTGATTCCTCTTCTT |
| TGFβ-2    | Forward: GCGGAGGTGTTTCCATCTA | Reverse: GGTGATCAGCTCCAGGTTTGACTT |
| TGFβ-3    | Forward: GATGGACCAATAGCAACCAACA | Reverse: ATGTGGCTGGAAAGGAGGAC |
| β-Actin   | Forward: CATCCCTAAAAGCCTTTAGGCC | Reverse: AGCAGGCTCAATACAGTCC |
| Gapdh     | Forward: TGGAGACGGCCATGTGGGAGG | Reverse: CGAAGGTCAAGACTGCGGAG |

into both flanks of five AhR+/+ and AhR−/− mice. Tumors were collected at 7 or 14 days after injection and their volume calculated as length × width2 × 0.4. After fixation in 4% paraformaldehyde, tissues were sectioned at 5 μm and processed for hematoxylin and eosin staining or immunofluorescence.

In Vivo Matrigel Plug Assays and Hemoglobin Content—Aliquots consisting of 500 μl of ice-cold solution of 70% Matrigel, 30% Hanks’ salt medium (Invitrogen), 30 units of heparin, and 300 ng of endothelial cell growth supplement were injected in duplicate into the flanks of at least four independent AhR+/+ and AhR−/− mice. One flank of each mouse was left as the control, and the other flank was used for the corresponding experimental treatments. In some experiments, VEGF-A164 (300 ng/ml), TGFβ (10 ng/ml), a neutralizing anti-VEGF-A antibody (200 ng/ml), or a neutralizing anti-TGFβ antibody (1 μg/ml) were added to the Matrigel plugs. After 7 days, plugs were harvested, photographed, homogenized in 1 ml ice-cold H2O, and centrifuged at 10,000 × g for 6 min at 4 °C. The amounts of hemoglobin released to the supernatants were determined using Drabkin’s reagent as indicated by the manufacturer (Sigma-Aldrich). Hemoglobin content is represented as mg/ml in 100 mg of Matrigel.

Isolation of Mouse Retina and Whole Mount Immunofluorescence—Retinas from five 3-day-old AhR+/+ and AhR−/− mice were isolated and processed as described previously (44). Briefly, eyes were collected and fixed overnight at 4 °C in Tris-buffered saline containing 4% paraformaldehyde. Retinas were dissected and permeabilized overnight at 4 °C in Tris-buffered saline containing 1% bovine serum albumin and 0.05% Triton X-100. After three washes in Tris-buffered saline, retinas were incubated overnight at 4 °C with isoelectric B4-FITC (Sigma-Aldrich) in Tris-buffered saline. Flat-mounted retinas were analyzed by fluorescence microscopy. Measurements were taken near the optic nerve, at the center of the retina.

Hematoxylin/Eosin Staining and Tissue Immunofluorescence—Hematoxylin/eosin staining of tumor tissues was performed as indicated previously (33). For immunofluorescence, tissue sections were rehydrated, blocked, and incubated with CD31 antibody overnight at 4 °C. After washing in phosphate-buffered saline, sections were incubated with a TRITC-labeled secondary antibody (Sigma-Aldrich), mounted in Mowiol, and photographed under fluorescence microscopy.

In Vitro Tube Formation and Co-culture Experiments—Matrigel (70-μl aliquots) was gelled at 37 °C for 1 h. Then, 105 MAECs were plated in OptiMEM (Invitrogen), and the formation of capillary-like structures was photographed 24 h later. For endothelial-mesenchymal co-culture experiments, AhR+/+ and AhR−/− immortalized T-FGM fibroblasts (33) were treated with mitomycin (Sigma-Aldrich) to inhibit cell proliferation. A sterile plastic ring was placed in a 35-mm tissue culture plate, and its interior volume was filled with Matrigel. After gelling, a monolayer of T-FGM AhR+/+ or T-FGM AhR−/− fibroblasts was seeded in the culture area outside the ring using OptiMEM. After 48 h, 105 HMEC-1 cells were plated on the Matrigel plug located inside the ring. Once the endothelial cells adhered to the Matrigel, the ring was removed, and 16 h later, the formation of capillary-like structures was photographed. Experiments were also performed using T-FGM-conditioned media rather than the cells themselves. T-FGM AhR+/+ and T-FGM AhR−/− fibroblasts growing at 30% confluence were cultured for 48 h in OptiMEM. Conditioned OptiMEM was then collected, processed as indicated previously (29), and used to analyze the formation of capillary-like structures as described above.

Measurement of Total Tube Length (TTL) and Calculation of Branching Points—TTL and branching points were measured in aortic ring explants and in Matrigel plugs by blinded analyses as described in Fig. 2A. Raw images were analyzed using ImageJ software to identify tubular structures. These images were subsequently employed to calculate TTL as the total length of the individual tubes present and branching as the number of bifurcations along the tubes (Fig. 2A, arrows).

Cell Viability and Cell Adhesion—After seeding for 16 h, 105 HMEC-1 cells were washed, fixed, and stained with crystal violet. Following extensive washing with water, cultures were photographed and the number of viable cells counted. For adhesion experiments, aliquots of 105 HMEC-1 cells were plated on gelled Matrigel plugs. After incubation at 37 °C for 1 h, cells were fixed, stained with crystal violet, and analyzed as indicated above.

Invasion in Matrigel Chambers and Wound Closure in Vitro—Invasion assays were done essentially as described (33). Briefly, 5 × 104 HMEC-1 cells were plated in the upper chamber of a Matrigel invasion Transwell (BD Biosciences) in either OptiMEM or fibroblast-conditioned medium. Dulbecco’s modified Eagle’s medium-F12 medium containing 10 ng/ml insulin-like growth factor-1 (Sigma-Aldrich) was added to the lower chamber as a chemoattractant. After 24 h, Transwells were washed with phosphate-buffered saline, fixed at 4 °C in 70% ethanol, treated for 15 min with RNase (10 ng/ml), and stained with
AhR Alters TGFβ and VEGF in Angiogenesis

AhR Alters TGFβ and VEGF in Angiogenesis

**FIGURE 1. AhR−/− mice have impaired tumor growth and inefficient angiogenesis.** A, B16F10 mouse melanoma cells were injected subcutaneously in the dorsal area of AhR+/+ and AhR−/− mice, and tumors were collected at 7 and 14 days after grafting. Tumor volume was calculated as length × width2 × 0.4/3; tumors were fixed, sectioned, and stained with hematoxylin and eosin or used to detect blood vessels by immunofluorescence with an antibody for the endothelial marker CD31. Vessels were counted at each time point, and the results corresponding to tumors at 14 days were represented. Note that hematoxylin and eosin images appear dark because of the high levels of melanin expressed by B16F10 melanoma cells. C, Matrigel was prepared and injected subcutaneously in the dorsal area of AhR+/+ and AhR−/− mice. After 7 days, the Matrigel plugs formed were removed, washed, and photographed, and the angiogenesis recruited in each genotype was quantitated by measuring the content in hemoglobin. Hemoglobin content is represented as mg/ml in 100 mg of Matrigel. Light microscopy was done at room temperature on a Nikon E-400 microscope equipped with a Nikon L16 camera. A × 10 objective (0.25 numeric aperture) was used on Eukitt-mounted sections. Immunofluorescence was done at room temperature on a Nikon TE2000U microscope equipped with a Nikon DS-S2 M digital camera. A ×10 objective (0.25 numeric aperture) was used on Mowiol-mounted sections. Data are shown as means ± S.D. Tumors were induced in both sides of five AhR+/+ and AhR−/− mice at each time point. Matrigel plugs were done in duplicate in at least four animals of each genotype.

propidium iodide. Cell invasion into Matrigel was analyzed in 5-μm steps using a Zeiss LSM 510 confocal microscope. Wound closure experiments were performed using HMEC-1 in the absence or presence of AhR siRNA essentially as described (25, 33).

**RNA Interference (RNAi) and Western Immunoblotting—** siRNA for AhR or unspecific scramble RNA (Dharmacon) was transiently transfected in HMEC-1 cells by electroporation using a MicroPorator MP-100 (Digital-Bio). siRNA or scramble RNA were transfected at 100 nM using three pulses of 10 ms with the voltage set at 1400 V. HMEC-1 cells were grown for 60 h and then trypsinized and used for in vitro Matrigel tube formation. A fraction of the transfected cells was used to analyze AhR expression by Western immunoblotting as described (45).

**Real-time PCR and ELISAs—** Quantitative real-time RT-PCR and data analyses for the expression of angiogenesis-related genes were done as described earlier (45). Oligonucleotide sequences used are indicated in Table 1. The amounts of active VEGF-A, TGFβ-1, and TGFβ-2 secreted by MAECs or T-FGM fibroblasts were determined using ELISA kits from Bender MedSystems following the manufacturer’s instructions. To activate latent TGFβ, conditioned medium was treated for 10 min at room temperature with 165 mM HCl and then neutralized with NaOH.

**Image Processing and Statistical Analyses—** Light and fluorescence microscopy measurements of aort rings and branching of capillary-like structures in Matrigel plugs were quantified in at least five random fields using ImageJ software. All determinations were done in triplicate in at least two independent experiments. Data are shown as mean ± S.D. Statistical comparison between experimental conditions was done using GraphPad Prism 4.0 software. A comparison between conditions was done using the unpaired Student’s t test, and multi-comparison was done using one-way analysis of variance followed by Dunn’s post test.

**RESULTS**

**Loss of AhR Expression Impairs Tumor Growth and Compromises Angiogenesis in Vivo—** In previous work studying the potential of AhR-null transformed fibroblasts to induce tumors in mice, we suggested that lack of AhR expression could compromise tumor development by reducing cell migration and/or angiogenesis (33). These results prompted us to analyze whether AhR modulates angiogenesis and how its expression in the endothelial and stromal compartments contributes to the process. We first performed xenografts of B16F10 mouse melanoma cells in the dorsal area of AhR+/+ and AhR−/− mice (Fig. 1A). Tumors produced by B16F10 cells in AhR-null mice had a significantly reduced volume as compared with those isolated...
AhR Alters TGFβ and VEGF in Angiogenesis

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shows that isolectin B4-FITC-labeled $\text{AhR}^{-/-}$ retinas had fewer branching points per field than retinas from $\text{AhR}^{+/+}$ mice. Altogether, these experiments suggest that AhR expression is relevant to promote and to maintain angiogenesis, probably through the modulation of tube formation and branching of endothelial cells. The causal role of AhR in tube formation and branching was demonstrated by down-modulating AhR expression with RNAi. Human endothelial cells (HMEC-1) constitutively expressed AhR, and transfection of siRNA against this receptor significantly decreased its protein levels with respect to unspecific scramble-transfected cells (Fig. 3C). AhR was shown to be responsible for endothelial tube formation and branching, because siRNA-transfected HMEC-1 cells mimicked the phenotype observed in $\text{AhR}^{-/-}$ MAECs (Fig. 3D). Reduced branching and impaired tube formation in the absence of AhR could be due to a decrease in cell viability and/or migration potential. However, down-modulation of AhR expression by siRNA did not significantly reduce viability or proliferation of HMEC-1 cells within a 72-h period (Fig. 3E). Further, wound healing in culture revealed that AhR siRNA did not affect the migration potential of HMEC-1 (Fig. 3F).

**VEGF-A Expression Is Down-modulated in $\text{AhR}^{-/-}$ MAECs: Role of Hypoxia and HIF-1α—VEGF is a major growth factor involved in angiogenesis and in vascular homeostasis (7, 9, 10). Because loss of AhR expression markedly altered angiogenesis in vivo and tubulogenesis and branching both in vivo and in culture, we decided to analyze whether the $\text{AhR}^{-/-}$ phenotype was related to altered VEGF expression and/or activity. Real-time RT-PCR was used to measure mRNA expression of the angiogenesis-related genes Hif-1α, Vegf-A, Vegf-B, Plgf, Vegfr-1,
AhR Alters TGFβ and VEGF in Angiogenesis

FIGURE 4. AhR<sup>+/+</sup> MAECs have reduced expression and activity of the proangiogenesis factor, VEGF. A, total RNA was purified from AhR<sup>+/+</sup> and AhR<sup>−/−</sup> MAECs and used to analyze Hif-1α, Vegf-A, Vegf-B, Plgf, Vegfr-1, and Vegfr-2 expression by real-time RT-PCR. Gene expression was normalized by Gapdh expression. Fold change represents the difference between AhR<sup>−/−</sup> MAECs and AhR<sup>+/+</sup> MAECs. The oligonucleotide sequences used to amplify each gene are indicated in Table 1. Determinations were done in triplicate in at least three MAEC cultures. B, mRNA expression for the Vegf<sub>120</sub>, Vegf<sub>164</sub> and Vegf<sub>188</sub> isoforms was determined by real-time RT-PCR. Differences in expression between AhR<sup>−/−</sup> and AhR<sup>+/+</sup> MAECs are indicated as fold change. The oligonucleotide sequences used to amplify each gene are indicated in Table 1. C, the amount of VEGF-A activity secreted by AhR<sup>−/−</sup> and AhR<sup>+/+</sup> MAEC and fibroblast cells was determined by ELISA in conditioned medium from each cell type and genotype. D, hypoxia was mimicked in AhR<sup>−/−</sup> and AhR<sup>+/+</sup> MAEC cultures by treatment with 100 μM CoCl<sub>2</sub> for 16 or 24 h, and Vegf expression was determined at the mRNA level as indicated under “Experimental Procedures.” Data were normalized and expressed as fold change with respect to solvent (DMSO)-treated cultures. In some experiments, MAECs were co-treated with 100 μM CoCl<sub>2</sub> and 5 μM 2-Me E2 for 24 h to induce HIF-1α degradation under hypoxic conditions. Vegf expression was normalized using β-actin instead of Gapdh to avoid potential effects of CoCl<sub>2</sub> on the expression of the latter gene. The experiments were done in triplicate in three MAEC cultures. Data are shown as means ± S.D.

and Vegfr-2. AhR<sup>−/−</sup> MAECs had a significant decrease in Vegf-A and, to a lesser extent, Vegf-B expression. Interestingly, the expression of the VEGF-A-target gene Hif-1α was also reduced in AhR<sup>−/−</sup> MAECs, suggesting that down-modulation of Vegf-A was functionally relevant (Fig. 4A). VEGF-A has several splice variants: VEGF<sub>188</sub>, which is slightly active and tightly bound to the extracellular matrix, and VEGF<sub>120</sub> and VEGF<sub>164</sub> which are diffusible forms able to induce angiogenesis in vivo (48, 49). Remarkably, AhR<sup>−/−</sup> MAECs had a significant down-modulation of VEGF<sub>164</sub> and VEGF<sub>120</sub> mRNAs as compared with AhR<sup>+/+</sup> MAECs, with VEGF<sub>164</sub> being the predominant isoform in these primary cells (Fig. 4B). Furthermore, the level of VEGF-A activity secreted to the medium (e.g. VEGF<sub>164</sub> and VEGF<sub>120</sub>) was markedly reduced in AhR<sup>−/−</sup> MAECs as compared with wild-type MAECs (Fig. 4C), suggesting that lower levels of VEGF-A could be relevant to the defective angiogenesis observed in AhR-null mice. In addition, the high level of VEGF<sub>188</sub> produced by AhR<sup>−/−</sup> MAECs did not seem to compensate for the impairment in angiogenesis, further suggesting that VEGF<sub>168</sub> is not functionally relevant and that the reduction in VEGF<sub>164</sub> and VEGF<sub>120</sub> activities was mainly responsible for the lower angiogenic potential of MAECs.

The reduced expression of HIF-1α in AhR<sup>−/−</sup> MAECs suggested that their deficiency in producing active VEGF and inducing angiogenesis could be due, at least in part, to an impaired response to hypoxia. To address this possibility, we treated AhR<sup>−/−</sup> and AhR<sup>+/+</sup> MAECs with CoCl<sub>2</sub> to mimic hypoxia and then determined Vegf mRNA expression. Hypoxia readily increased Vegf expression with time in AhR<sup>−/−</sup> MAECs, whereas no significant induction was observed in AhR<sup>+/+</sup> MAECs (Fig. 4D). The causal role of AhR in HIF-1α-mediated control of Vegf expression was further analyzed using HMEC-1 cells, which were found to respond to AhR down-modulation by RNAi (see Fig. 3). Transfection of HMEC-1 cells with AhR siRNA reduced the mRNA expression of both Hif-1α and Vegf-A (Fig. 5A). Accordingly, treatment with 2-Me E2 decreased Vegf levels in mock-transfected (scramble) but not in AhR siRNA-transfected HMEC-1 cells (Fig. 5B). Thus, reduced angiogenesis in AhR<sup>−/−</sup> endothelial cells seems to be the result of lower VEGF-A production due to decreased HIF-1α expression.

Based on these results, we next used recombinant VEGF protein (purified VEGF<sub>164</sub> and R&D Systems) and an inhibitory anti-VEGF antibody (neutralizes VEGF<sub>164</sub> and VEGF<sub>120</sub>; Sigma-Aldrich) in an attempt to modulate angiogenesis in culture and in vivo. The addition of anti-VEGF antibody to AhR<sup>+/+</sup> aortic rings decreased the TTL to values similar to those found in AhR<sup>−/−</sup> aortic rings, whereas treatment of AhR<sup>−/−</sup> rings with recombinant VEGF protein did the opposite and rescued TTL levels to AhR<sup>+/+</sup> values (Fig. 6A). In vivo, the addition of anti-VEGF antibody to Matrigel plugs implanted in AhR<sup>+/+</sup> mice markedly reduced angiogenesis, whereas recombinant VEGF protein induced a modest, although very reproducible increase in blood vessels recruitment in plugs injected in AhR<sup>−/−</sup> mice (Fig. 6B). Increased Secretion of TGFβ Activity by Stromal Fibroblasts Also Contributes to Defective Angiogenesis in AhR<sup>−/−</sup> Mice—TGFβ is a cytokine with a relevant role in angiogenesis that
AhR Alters TGFβ and VEGF in Angiogenesis

cooperates functionally with VEGF in endothelial cell function (16, 17, 22). Because we had found previously that stromal fibroblasts from AhR−/− mice secrete increased levels of active TGFβ (25, 29), here we established co-cultures of Matrigel-embedded HMEC-1 cells and fibroblasts isolated from AhR+/+ or AhR−/− mice (Fig. 7A). HMEC-1 cells co-cultured with AhR−/− fibroblasts lost their ability to form tubes as compared with the same endothelial cells co-cultured with AhR+/+ fibroblasts (Fig. 7B). Moreover, such an effect on the endothelial cells was produced by a soluble and secreted molecule, because conditioned medium (CM) from AhR−/− fibroblasts mimicked the effects induced by the fibroblasts themselves (Fig. 7C).

AhR−/− fibroblasts expressed slightly increased levels of Tgfβ-1 and ~2-fold more Tgfb-2 mRNA than wild-type cells as determined by real-time RT-PCR (Fig. 8A, left). Nevertheless, TGFβ has to be secreted and activated in the extracellular matrix (15, 50) to exert its effects, and because AhR-null fibroblasts produce elevated levels of TGFβ activity (25, 29, 37, 51), we determined the amounts of active and total TGFβ-1 and TGFβ-2 released by AhR+/+ and AhR−/− fibroblasts by ELISA. We found that the total amount of TGFβ-1 secreted to the medium was markedly higher than that of TGFβ-2 in both genotypes (Fig. 8A, center and right). Importantly, AhR−/− fibroblasts released higher levels of both active and total TGFβ-1 than AhR+/+ cells, although no significant differences were observed with respect to active TGFβ-2 (Fig. 8A, center and right). Thus, increased production of TGFβ-1/2 by stromal fibroblasts could contribute to the inhibition of angiogenesis. Consistent with this hypothesis, the addition of recombinant TGFβ to medium conditioned by AhR+/+ fibroblasts inhibited tube formation by HMEC-1 cells, whereas a neutralizing anti-TGFβ antibody added to medium conditioned by AhR−/− fibroblasts increased tube formation (Fig. 8B). These detrimental effects on angiogenesis were not due to an inhibitory activity of the AhR−/− conditioned medium or the TGFβ recombinant protein on HMEC-1 viability (Fig. 8C) or adhesion to Matrigel (Fig. 8D).

Because angiogenesis requires endothelial cell invasion of the surrounding tissue, we next used HMEC-1 cells as a model to analyze their invasion potential in the presence of medium conditioned by AhR+/+ fibroblasts and to determine whether TGFβ would contribute to such effect. HMEC-1 cells were plated in Matrigel-coated Transwells, and their invasive ability was determined by confocal microscopy. We found that the addition of medium conditioned by AhR+/+ fibroblasts to the upper chamber significantly inhibited HMEC-1 invasion as compared with endothelial cells treated with medium from wild-type fibroblasts (Fig. 9A). Furthermore, the addition of recombinant TGFβ to the medium conditioned by AhR+/+ fibroblasts decreased HMEC-1 invasion, whereas a neutralizing anti-TGFβ antibody did the opposite, increasing HMEC-1 invasion in the presence of medium conditioned by AhR−/− fibroblasts (Fig. 9A). Consistently, recombinant TGFβ was able to decrease blood vessel recruitment to Matrigel plugs injected in AhR+/+ mice, whereas a neutralizing anti-TGFβ antibody enhanced vessel recruitment to Matrigel inserts in AhR−/− mice (Fig. 9B). Importantly, VEGF and TGFβ acted together in AhR-dependent angiogenesis, because co-treatment of AhR+/+ mice with anti-VEGF antibody plus TGFβ decreased blood vessel recruitment to Matrigel plugs, whereas the addition of VEGF plus anti-TGFβ antibody increased angiogenesis in Matrigel plugs implanted in AhR−/− mice (Fig. 9B). This possible interaction between VEGF and TGFβ activities in endothelial cells is also supported by the observation that treatment of AhR−/− MAECs with exogenous TGFβ further reduced their basal low level of VEGF-A secretion (Fig. 9C). Altogether, these data strongly suggest that TGFβ activity secreted to the extracellular medium by stromal fibroblasts contributes significantly to angiogenesis and that overactivation of TGFβ in the absence of AhR is a relevant factor in explaining their angiogenesis deficiency. In addition, the fact that an increase in VEGF cooperated with decreased TGFβ activity in improving angiogenesis in AhR−/− mice (or the opposite in wild-type mice) further supports a functional interaction between endothelial and stromal cells in vessel recruitment and underlines AhR as a regulator of the process.
AhR also has a role in the cardiovascular system. The analysis of AhR-null mice has shown that, in the absence of xenobiotics, loss of AhR expression induces cardiac hypertrophy (26) with hypertension and elevated plasma levels of angiotensin II and endothelin-1 (53). In addition, AhR−/− mice have hepatic vascular defects characterized by the failure to resolve the embryonic portosystemic shunt (23, 24). Moreover, we have suggested that the lower efficiency of transformed AhR−/− fibroblasts to generate tumor xenografts in vivo could be due to an impaired ability to induce angiogenesis (33). Because AhR seemed relevant to vascular homeostasis and angiogenesis, we decided to analyze further whether AhR expression compromises endothelial cell function and angiogenesis. 

Trying to accomplish that goal, experiments were performed using in vivo animal models, ex vivo tissue culture explants, and primary cultures of rodent and human endothelial cells. A major conclusion from our current work is that angiogenesis requires not only AhR-dependent expression of VEGF-A by endothelial cells but also AhR-dependent down-modulation of TGFβ activity by stromal fibroblasts. The mechanisms by which AhR signaling modulates angiogenesis appear complex, as a recent report has revealed that AhR-null mice have increased response to ischemia-induced angiogenesis (54). That study suggests that because the aryl hydrocarbon receptor nuclear translocator (ARNT/HIF-1α) is a common partner for both AhR and HIF-1α (55, 56), and given that ischemia increases the expression of VEGF, ARNT, and HIF-1α to a greater extend in AhR−/− than in AhR+/+ mice, the lack of competition between AhR and HIF-1α for common co-regulators in AhR-null mice would result in improved angiogenesis due to overactivation of the HIF-1α signaling pathway. Although this is a plausible hypothesis, and ARNT has an evident role in angiogenesis (57, 58), other studies have shown that activation of either AhR- or HIF-1α-dependent signaling does not inhibit the activity of the other pathway as a
AhR Alters TGFβ and VEGF in Angiogenesis

result of competition for ARNT (59). Our data in fact indicate that genetic ablation of AhR in mouse endothelial cells or its down-modulation by RNAi in the human endothelium decreases HIF-1α levels, thus making the expression of HIF-1α AhR-dependent in these cell types. Therefore, the question remains as to how AhR participates in endothelial cell function under physiological and stress conditions and which are the regulatory pathways involved. This study focuses on the role of AhR in angiogenesis under normal cell conditions and identifies VEGF and TGFβ as relevant growth factors that interact functionally in an AhR-dependent manner.

In agreement with our prior study (33) and with reports showing that constitutive AhR activation enhances tumor growth (60, 61), we have found that lack of AhR expression in mice severely impairs their capacity to support the growth of melanoma tumors, suggesting that an AhR-competent microenvironment is needed for such an effect. The fact that melanoma tumors from AhR−/− mice had decreased blood vessel content provided additional support for AhR in tumor angiogenesis. Indeed, blood vessels from mice lacking AhR expression were much less capable of invading angiogenesis-promoting Matrigel plugs. The sprouting of expanding and invading vessels markedly influences angiogenesis through branching of the endothelial cells located at the tip end and tube formation by trailing stalk cells (9).

**FIGURE 7. Co-culture of human endothelial HMEC-1 cells with AhR−/− fibroblasts inhibits tube formation.** (A) Scheme showing the experimental system used to co-culture HMEC-1 cells with fibroblasts. B, HMEC-1 cells grown in Matrigel plugs were co-cultured with AhR+/+ or AhR−/− fibroblasts, and their ability to form tubes was determined by measuring TTL as described (47). C, HMEC-1 cells were cultured in Matrigel plugs in the presence of medium conditioned (C.M.) by AhR+/+ or AhR−/− fibroblasts, and TTL was determined as above. Light microscopy was done at room temperature on a Nikon E-400 microscope equipped with a Nikon L16 camera with a ×10 objective (0.25 numeric aperture). The experiments were performed in four HMEC-1 cultures for each experimental condition. Data are shown as means ± S.D.
AhR Alters TGFβ and VEGF in Angiogenesis

The addition of recombinant TGFβ or neutralizing anti-TGFβ antibody to medium conditioned by AhR+/− fibroblasts modulates angiogenesis of HMEC-1 cells. A, expression of Tgfβ-1, Tgfβ-2, and Tgfβ-3 isoforms was analyzed in AhR+/+ and AhR−/− fibroblasts by real-time RT-PCR using the oligonucleotides indicated in Table 1. Secretion of latent and active TGFβ-1 and TGFβ-2 proteins by AhR+/+ and AhR−/− fibroblasts to the culture medium was determined by specific ELISA as indicated under "Experimental Procedures." To activate latent TGFβ, conditioned medium was treated for 10 min at room temperature with 165 mM HCl and then neutralized with NaOH. B, HMEC-1 were plated in Matrigel and treated with CM from AhR+/+ or AhR−/− fibroblasts, with AhR+/+ CM plus 10 ng/ml recombinant TGFβ, or with AhR−/− CM plus 1 μg/ml neutralizing anti-TGFβ antibody (Ab). TPL was determined as indicated (47). Light microscopy was done at room temperature on a Nikon E-400 microscope equipped with a Nikon L16 camera with a 10 objective (0.25 numeric aperture). The experiments were performed in three HMEC-1 cultures for each experimental condition. Data are shown as means ± S.D. C, viability of HMEC-1 cells was determined under the same experimental conditions as those used in B. D, cell adhesion was quantitated in HMEC-1 cells under the experimental conditions used in B. Determinations were done in triplicate in at least two different HMEC-1 cultures. Data are shown as means ± S.D.
AhR Alters TGFβ and VEGF in Angiogenesis

Reduced angiogenesis in AhR\textsuperscript{+/+} mice under the same experimental conditions. The mechanism by which AhR modulates angiogenesis through VEGF is in large part unknown, although several sets of data suggest the contribution of hypoxia and HIF-1α. Firstly, MAECs growing under hypoxia-mimicking conditions by CoCl\textsubscript{2} treatment were able to induce Vegf but only if they expressed AhR. Secondly, inducing HIF-1α degradation by 2-Me E2 blocked CoCl\textsubscript{2}-dependent Vegf induction in AhR\textsuperscript{+/+} but not in AhR\textsuperscript{−/−} endothelial cells. Thirdly, down-modulation of AhR by siRNA in HMEC-1 cells decreased Hif-1α and Vegf expression, although 2-Me E2 inhibited Vegf levels only in AhR-expressing cells. Finally, a previous study has indicated that AhR activation by dioxin in thymic epithelial cells induces Vegf expression as did CoCl\textsubscript{2} (63). Altogether, we propose that AhR expression in endothelial cells maintains the production of active VEGF isoforms, at least in part through HIF-1α, and that alteration of such mechanism contributes to defective angiogenesis in AhR-null mice.

Nonetheless, angiogenesis is not only regulated through endothelial cells but also involves functional interactions with different components of the stroma (13). Among them, TGFβ is considered an important growth factor able to inhibit angiogenesis (16, 64). Considering our previous studies showing that AhR modulates TGFβ activity in primary and immortalized stromal fibroblasts (25, 29, 34), we sought to analyze whether the increase in TGFβ activity by AhR\textsuperscript{−/−} fibroblasts could add to the deficient angiogenesis observed in AhR-null mice. Co-culture of human HMEC-1 cells with AhR\textsuperscript{−/−} fibroblasts severely impaired their angiogenesis potential. Because the same effects were found by using conditioned medium from AhR\textsuperscript{−/−} fibroblasts, we concluded that a secreted angiogenesis inhibitor should be involved. TGFβ emerged as a potentially relevant candidate because AhR\textsuperscript{−/−} fibroblasts expressed higher levels of Tgfβ-1 and Tgfβ-2 and, more importantly, because these cells secreted increased amounts of active and total cytokine, particularly TGFβ-1. This hypothesis was confirmed by experiments per-
formed in the presence of recombinant TGFβ protein or a neutralizing anti-TGFβ antibody. Thus, the addition of TGFβ inhibited tube formation in HMEC-1 cells and angiogenesis in Matrigel plugs implanted in AhR+/−/− mice, whereas a neutralizing anti-TGFβ antibody did the opposite, promoting both tube formation in human endothelia and angiogenesis in Matrigel plugs injected in AhR−/− mice. In agreement, previous studies have also shown that TGFβ or its neutralizing antibody either block or stimulate embryonic vasculogenesis (20). Thus, AhR expression in stromal fibroblast also contributes to angiogenesis by regulating the production of active TGFβ in the extracellular matrix. Several mechanisms have been proposed to explain how AhR inhibits TGFβ expression/activation in fibroblast cells. They include transcriptional repression of the Tgfβ-1 and Tgfβ-2 genes (this work and Ref. 65), down-regulation of the latent TGFβ-binding protein that binds TGFβ to the extracellular matrix (29, 51), and inhibition of extracellular proteases (e.g. plasminogen activators/plasmin and elastase) known to be responsible for TGFβ activation (37). We suggest that alteration of these mechanisms in AhR lacking fibroblasts could result in TGFβ overactivation, which, in turn, could cooperate with decreased VEGF activity to inhibit angiogenesis. It is also possible that the inhibitory effects on angiogenesis of stroma-derived TGFβ could take place through reduction of the invasive properties of the endothelium, suggesting that whereas endothe-lia-derived VEGF modulates angiogenesis by regulating branching, stromal TGFβ could exert its effects by affecting cell invasion.

In addition to their individual roles as angiogenesis regulators, our study provides experimental support for the proposed interaction between VEGF and TGFβ in the control of vessel formation (9, 17, 20). Indeed, VEGF and TGFβ had opposing effects on angiogenesis in such a way that their coordinated secretion by AhR-null endothelial and fibroblast cells probably determines the lower angiogenesis present in AhR−/−/− mice. Remarkably, the intracellular AhR receptor seems to be a common partner, able to regulate VEGF and TGFβ activities in the extracellular medium and, ultimately, endothelial cell branching and tubulogenesis under basal and pathological conditions. In this context, the fact that natural AhR antagonists such as resveratrol can inhibit tumor angiogenesis (66) by interfering with VEGF expression and HIF-1α accumulation in human squamous carcinoma SCC-9 and hepatoma HepG2 cells (67) identifies AhR as novel potential therapeutic target to block angiogenesis in certain diseases such as cancer. From a mechanistic point of view, it is clear that AhR modulates TGFβ activity by regulating its location and activation in the ECM through the transcriptional control of the latency protein LTBP-1 and the activity of ECM proteases (25, 37, 51) and by controlling the expression of TGFβ-related genes (68, 69). Regarding the regulation of VEGF-A, our data suggest the involvement of HIF-1α, a major regulatory protein for this growth factor (70, 71) that is also down-modulated in AhR−/−/− MAECs. Alternatively, because the Vegf-A promoter contains xenobiotic response elements for AhR binding, AhR can also maintain constitutive VEGF expression as shown previously for the target genes Cyp1a2 (39), Vav3 (72), and p27Kip1 (73).

In summary, this study provides additional support for the widely accepted implications for AhR in cell physiology and pathology. We propose here that the coordinated control by AhR of VEGF activity in endothelial cells and TGFβ in fibroblasts represents a mechanism that can integrate these two growth factors in the progression of angiogenesis. It will be of interest to determine whether AhR, VEGF, and TGFβ are coordinately regulated during tumor angiogenesis.

References—We are very grateful to Drs. Carlos Lopez-Otin and Xose Puente (Universidad de Oviedo, Spain) for kindly providing the B16F10 mouse melanoma cell line. The HMEC-1 cell line was generously provided by Dr. Edwin Ades (Centers for Disease Control, Atlanta, GA). We are also grateful to Dr. Xose R. Bustelo (Centro de Investigación del Cancer, Salamanca, Spain) for assistance with confocal microscopy and greatly appreciate the expert technical assistance of Edurne San Vicente.

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