The Transforming Growth Factor-β Family Members Bone Morphogenetic Protein-2 and Macrophage Inhibitory Cytokine-1 as Mediators of the Antiangiogenic Activity of N-(4-Hydroxyphenyl) Retinamide

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Abstract Purpose: Tumor growth appears to be an angiogenesis-dependent process. N-(4-hydroxyphenyl)retinamide (fenretinide; 4HPR) has been found to inhibit and/or prevent tumor growth under diverse conditions. Although 4HPR is antiangiogenic, the molecular mechanisms of this effect remain largely unknown.

Experimental Design: Endothelial cells were treated with 4HPR in vitro to study the effects on migration, invasion, and organization as well as gene expression by microarray and quantitative PCR studies. In vivo angiogenesis was evaluated in the Matrigel model.

Results: 4HPR treatment substantially modified the biological activities of endothelial cells, repressing their capacity to migrate, invade, and organize into capillary-like structures. The inhibition of invasion induced by 4HPR was also associated with decreased activities of the metalloproteases matrix metalloproteinase-2 and CD13/APN. Using oligonucleotide microarrays, we observed that bone morphogenetic protein-2 and macrophage inhibitory cytokine-1, two multifunctional cytokines of the transforming growth factor-β family that regulate the growth, differentiation, apoptosis, and matrix accumulation of a variety of cells, are up-regulated in vitro by 4HPR. Both these molecules specifically inhibited endothelial cell growth, migration, and invasion in vitro and suppressed angiogenesis in the Matrigel plug assay in vivo. Blocking antibodies to bone morphogenetic protein-2 were able to reverse the suppressive effects of 4HPR in vitro and in vivo.

Conclusions: These data support the conclusion that 4HPR inhibits tumor growth by repression of new vessel growth and identify novel points of regulation of angiogenesis in transforming growth factor-β family proteins.

Angiogenesis, the creation of a new blood supply network from preexisting blood vessels, mainly depends on the activation, proliferation, and migration of endothelial cells. Tumor growth, invasion, and metastasis are angiogenesis-dependent processes driven by angiogenic stimuli that activate endothelial cells and induce proteolytic enzymes needed to cleave the extracellular matrix. The balance between proangiogenic and antiangiogenic factors normally tightly regulates angiogenesis (1). The relationship between tumor progression and angiogenesis is now well documented, and the modulation of blood vessels growth is an effective means of limiting or controlling tumor growth and spread (2, 3). Consequently, increasing importance has been given to the search for suitable targets that modulate angiogenesis (4, 5).

Retinoids have been shown to exert chemopreventive and antitumor activities in a variety of normal and malignant cells (6–8), and several studies have indicated that retinoid treatment is associated with an inhibition of angiogenesis and with a decreased vascular response in vitro and in vivo (9–12). The vitamin A analogue N-(4-hydroxyphenyl)retinamide (fenretinide; 4HPR) has been shown to inhibit breast carcinogenesis in preclinical studies (13), and premenopausal women treated with 4HPR showed a significantly lower incidence of new breast cancers than the corresponding control group in a clinical trial (14). These patterns are typical of antiangiogenic agents that prevent further development of occult metastases even if unable to control growth of the original primary tumor (15). Moreover, 4HPR has been shown to limit endothelial cell growth in the absence of apoptosis in vitro (16) and to inhibit angiogenesis in the chick chorioallantoic membrane (17, 18) and in the Matrigel plug assays in vivo (16). Further, we have reported previously that...
4HPR administration potently inhibited growth in vivo of established Kaposi’s sarcoma xenotransplants, a highly vascularized tumor, in early intervention protocols (16).

Here, we show that 4HPR potently inhibited the organization, migration, and invasion in vitro of human umbilical vein endothelial cells (HUVEC). These activities were associated with decreased release of matrix metalloproteinase (MMP)-2 and CD13/APN activities. Microarray transcriptome analyses indicated that 4HPR modulated a restricted set of genes coding for signaling molecules in endothelial cells, and we show that two of these genes, bone morphogenetic protein-2 (BMP-2) and macrophage inhibitory cytokine-1 (MIC-1), members of the transforming growth factor-β (TGF-β) family, are capable of directly controlling endothelial cell migration and invasion in vitro and angiogenesis in vivo. Our studies identify novel points of regulation of angiogenesis by 4HPR that further support its use as a chemopreventive or therapeutic agent to specifically target tumor angiogenesis.

Materials and Methods

Cell culture and chemicals. HUVECs were obtained from the American Type Culture Collection (Rockville, MD) and cultivated on gelatin-coated plates (1.5% in PBS) in M199 containing 10% heat-inactivated FCS, 100 μg/ml heparin, 10 ng/ml acidic fibroblast growth factor, 10 ng/ml basic fibroblast growth factor, 10 ng/ml epidermal growth factor, and 10 μg/ml hydrocortisone. Human microvascular endothelial cells neonatal/adult dermis and human aortic endothelial cells were from Cascade (Portland, OR) and cultivated in MVGS supplemented medium 131 (human microvascular endothelial cells neonatal/adult dermis) or LSGS supplemented medium 200 (human aortic endothelial cells). Human microvascular endothelial cells lung was obtained from Cambrex (Caravaggio, Italy) and grown in EGM-2-MV medium (Cambrex). 4HPR (Calbiochem, La Jolla, CA) was dissolved in absolute ethanol at a stock concentration of 10 mmol/L and stored in aliquots at −70°C. Human recombinant BMP-2 and MIC-1 and the BMP-2 Quantikine ELISA kit from R&D (Minneapolis, MN), anti-β-tubulin antibodies were from Sigma (Milan, Italy), peroxidase-conjugated secondary antibodies from Amersham (Milan, Italy), and irrelevant mouse IgG1 antibodies were from DAKO (Denmark). Polyclonal anti-MIC-1 antibodies and monoclonal blocking anti-BMP-2 (IgG1 clone 100230) antibodies were from R&D.

Chemotaxis and invasion assays. Chemotaxis and chemoinvasion assays on HUVECs were carried out in Boyden chambers as described previously (19). Cells (5 × 10⁴) were extensively washed with PBS, resuspended in serum-free medium, and placed in the upper compartment with or without 4HPR, BMP-2, or MIC-1 or the appropriate antibody. In parallel experiments, trypan blue exclusion under these conditions showed no decreased cell viability compared with controls. The two compartments of the Boyden chamber were separated by a 12 μm pore-size polycarbonate filters coated with 5 μg/ml collagen IV for the chemotaxis assay or with Matrigel (15 μg/ml), a reconstituted basement membrane, for the invasion assay. Supernatants from NIH3T3 cells (NIH3T3-CM) were used as chemoattractants in the lower chamber. After 6 hours of incubation at 37°C in 5% CO₂, the filters were recovered, the cells on the upper surface were mechanically removed, and those on the lower surface were fixed and stained. The migrated cells were counted in 5 to 10 fields for each filter under a microscope. The experiments were done in triplicate and repeated thrice.

Matrigel morphogenesis assay. A 24-microwell plate, prechilled at −20°C, was carefully filled with 300 μL/well of liquid Matrigel (10 mg/mL) at 4°C with a prechilled pipette, avoiding bubbles. The Matrigel was polymerized for 1 hour at 37°C, and HUVECs (70,000 per well) were suspended in regular medium in the absence or presence of different concentrations of 4HPR, BMP-2, or MIC-1 and carefully layered on the top of polymerized Matrigel. The effects on the growth and morphogenesis of endothelial cells were recorded after 6 and 24 hours with an inverted microscope (Leitz DM-IRB) equipped with CCD optics and a digital analysis system. Gelatin zymography and activity of CD13/APN. Supernatants of cells at the end of invasion assays conducted in the absence or in the presence of different concentrations of 4HPR, BMP-2, or MIC-1 were removed and remaining cells were pelleted by centrifugation. The protein content was measured by the Bradford method (Bio-Rad, Hercules, CA), and gelatin zymography was done as described previously (20). Briefly, SDS-PAGE gels were prepared containing copolymerized gelatin at a final concentration of 1.6 mg/mL. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 1 hour to remove SDS and incubated for 18 hours at 37°C in collagenase buffer [40 mmol/L Tris, 200 mmol/L NaCl, 10 mmol/L CaCl₂ (pH 7.5)]. Gels were then stained in 0.5% Coomassie brilliant blue followed by destaining. The enzyme-digested regions were observed as white bands against a blue background.

Cell surface aminopeptidase activity in HUVECs was measured after incubating cells with various concentration of 4HPR, BMP-2, or MIC-1 for 4, 16, and 24 hours in complete medium. Cells (40,000 per well) were washed thrice with PBS and incubated 1 hour at 37°C with 2 mL of 100 μmol/L l-alanine-4-methyl-7-coumarinylamide trifluoracetate (Fluka, Milan, Italy) in 10 mmol/L HEPES-buffered PBS (pH 7.2) containing 0.1% bovine serum albumin. The development of the fluorescent product was measured with a fluorometric plate reader (excitation wavelength, 360 nm; emission wavelength, 465 nm).

Preparation of RNA, cRNA, and GeneChip microarray analysis. Total RNAs were isolated from endothelial cells treated for 5 hours with 5 μmol/L 4HPR or from control cells using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. cDNA synthesis was done using T7-(dT)₂₄ oligo primers and the Custom SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen, Irvine, CA). Double stranded cDNAs were extracted with phenol/ chloroform/isoamyl alcohol (25:24:1), ethanol precipitated, and used to prepare cRNAs using the Bioarray High-Yield RNA Transcription kit (Affymetrix, Santa Clara, CA) according to the manufacturer’s instructions. cRNAs were purified using the RNeasy Mini kit, controlled by agarose gel electrophoresis, and subjected to fragmentation for 35 minutes at 94°C in fragmentation buffer [40 mmol/L Tris-acetate (pH 8.1), 100 mmol/L CH₃COOK, 30 mmol/L Mg(CH₃COO)₂·H₂O]. Labeled cRNA was used for screenings of GeneChip Human Genome U95Av2 arrays (Affymetrix). The experiment consisted of three independent experiments comparing 4HPR-treated and control using HUVECs from different donors (biological replicates). Data were collected using an Affymetrix scanner. The data collected were elaborated using the GCRMA (21) package of Bioconductor 1.9 (22). The intensity data were further analyzed using GeneSpring 6.1.1. Functional annotations were obtained from NetAffx (Affymetrix).

Real-time PCR. Total RNAs were isolated from controls and cells treated with 1 or 5 μmol/L 4HPR and reverse transcription was done with oligo(dT) primers. In some cases, the cells were pretreated with 5 μg/mL cycloheximide for 2 hours before, and maintained during, 4HPR treatment to block protein synthesis. mRNA expression was analyzed by quantitative real-time reverse transcription-PCR by using the following specific primers: BMP-2 sense 5’-GGAGGACCTCAGCTGACGGC-3’ and antisense 5’-AGAAGAATCCTCCGGTGTGTT and MIC-1 sense 5’-CGACCGAGAAGTCTGAGT-3’ and antisense 5’-GGAGGAGA-3’. The other primer sequences are available on request. The relative expression of each gene was assessed in comparison with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase amplified with the primers: sense 5’-GGAGGTAGGCTGCGAGT-3’ and antisense 5’-CATCGGTTGCAATCATGGAGA. cDNAs were amplified for 50 cycles using iQ Supermix (Bio-Rad) containing the intercalating agent SYBR Green in a two-step amplification scheme (95°C, 15 seconds and 60°C, 30 seconds). Fluorescence was measured

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during the annealing step on a Bio-Rad iCycler iQ instrument. Blank controls that did not contain cDNA were run in parallel. All samples were run in triplicate. Following amplification, melting curves with 80 steps of 15 seconds and a 0.5 °C temperature increase per step were done to control for amplicon identity. Relative expression values with SEs and statistical comparisons (unpaired two-tailed t test) were obtained using Qgene software (23).

Cell proliferation and apoptosis assays. For growth analysis, 2,500 HUVECs per well were seeded in gelatin-coated 96-well plates and grown in complete medium or treated with the different molecules at different concentrations. Medium was changed every 48 hours. The number of viable cells was measured over time using the crystal violet assay. Briefly, after fixation and staining in a solution of 0.75% crystal violet, 0.35% NaCl, 32% ethanol, and 3.2% formaldehyde, the cells were dissolved in 50% ethanol and 0.1% acetic acid and read in a microtiter spectrophotometer at 595 nm.

To measure any enrichment of cytoplasmic histone-associated DNA fragments after BMP-2 or MIC-1 induced cell death, a commercially available kit was employed (Cell Death Detection ELISA, Roche, Mannheim, Germany) using 24-well plates seeded with 30,000 HUVECs per well and grown in complete medium for 48 and 96 hours with various concentrations of the two molecules.

Bone morphogenetic protein-2 and macrophage inhibitory cytokine-1 immunodetection. BMP-2 protein released into the medium by HUVECs was measured using a commercial ELISA kit (R&D) following the manufacturer’s instructions. The supernatants from cells grown in complete medium without or with 5 µmol/L 4HPR were collected after 24 or 48 hours. The assay was run in triplicate and repeated several times with similar results. Cellular proteins were isolated from control and 4HPR-treated HUVECs in lysis buffer [6 mol/L urea, 62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% glycerol] or radioimmunoprecipitation assay buffer. Samples were subjected to electrophoresis and transferred to Hybond enhanced chemiluminescence membranes (Amersham). Proteins were examined using specific antibodies to BMP-2 and MIC-1. The membranes were subsequently incubated for 1 hour with peroxidase-labeled secondary antibodies (Amersham) and specific complexes were revealed by enhanced chemiluminescence solution (Amersham).

In vivo angiogenesis. We used the Matrigel sponge model of angiogenesis as described previously (24). VTH (100 ng/ml vascular endothelial growth factor, 2 ng/ml tumor necrosis factor-α, and heparin) either alone or in combination with 4HPR (5 µmol/L final concentration) or BMP-2 or MIC-1 (both at 100 ng/mL) were added to unpolymerized liquid Matrigel at 4 °C and the mixture was brought to a final volume of 600 µL. Neutralizing anti-BMP-2 antibodies were used at 15 µg/mL; as a control, irrelevant isotype-matched mouse IgG1 antibodies were used in excess. The Matrigel suspension was then slowly injected s.c. into the flanks of C57/BL6 male mice (Charles River, Lecco, Italy) with a cold syringe. At body temperature in vivo, the Matrigel quickly polymerizes to form a solid gel. After 4 days, gels from all groups were collected and weighed. Samples were minced and diluted in water to measure the hemoglobin content with a Drabkin reagent kit (Sigma).

Results

In vitro effects of N-(4-hydroxyphenyl)retinamide on endothelial cells. The effects of 4HPR at different concentrations on endothelial cell migration and invasion were tested in vitro. Fibroblast conditioned medium (NIH3T3-CM), which contains a mixture of molecules able to stimulate the migration of invasives, was used as chemoattractant in these experiments. HUVECs readily migrated (Fig. 1A) and invaded through Matrigel (Fig. 1B) in response to NIH3T3-CM, whereas migration in the absence of a chemoattractant (serum-free medium) was limited. The addition of 4HPR in the upper chambers had prominent effects on the migration and invasion of endothelial cells, with significant inhibition at 1 and 5 µmol/L (P < 0.001, two-tailed t test), whereas concentrations of ≤100 nmol/L had no effect (Fig. 1A and B).

Matrigel morphogenesis assay. Chemotaxis and chemoinvasion assays describe the initial steps of endothelial cell activation, whereas the morphogenesis assay on Matrigel is indicative of the ability of endothelial cells to differentiate into capillary-like networks. Untreated HUVECs produced the typical anastomosed cellular network 6 hours after plating (Fig. 2). Treatment with 0.1 µmol/L 4HPR partially inhibited the formation of these capillary-like structures, and higher

![Fig. 1](https://www.aacrjournals.org/clinics/canres/2005/11/15/4612/fig1.png)

**Fig. 1.** Inhibition of HUVEC migration (A) and invasion (B) by increasing doses of 4HPR. Serum-free medium (SFM) and supernatants of NIH3T3 fibroblasts were used as negative and positive controls, respectively. Experiments were done in triplicate and repeated thrice. 4HPR (1 and 5 µmol/L) inhibited migration and invasion. Columns, mean; bars, SD. ***, P < 0.001 (two-tailed t test).
concentrations completely blocked the growth and morphogenic organization of endothelial cells on Matrigel (Fig. 2).

**Effect of N-(4-hydroxyphenyl)retinamide on metalloprotease activity associated with human umbilical vein endothelial cells.** Because invasion of basement membranes requires metalloproteases essential to the invasive process, we tested whether 4HPR could inhibit the activities associated with these enzymes. The HUVECs stimulated to invade produced a characteristic gelatinase activity corresponding to the 62-kDa activated form of gelatinase A (MMP-2). 4HPR did not have direct effects on the enzymatic activity of MMP-2 or MMP-9 when added directly to the buffer of the gelatin zymography (data not shown). However, zymographic evaluation of supernatants of cells obtained from the invasion assays described above showed a markedly decreased MMP-2 activity produced by HUVECs with increasing concentrations of 4HPR. 4HPR inhibited the gelatinase activity in a dose-dependent manner with maximal inactivation at 1 \( \mu \)mol/L (Fig. 3A).

We also tested the activity of the aminopeptidase CD13/APN, which has been reported to be an important regulator of endothelial morphogenesis during angiogenesis (25). CD13/APN activity was decreased in a dose-dependent manner in HUVECs exposed to increasing concentrations of 4HPR. 4HPR inhibited the gelatinase activity in a dose-dependent manner with maximal inactivation at 1 \( \mu \)mol/L (Fig. 3A).

**N-(4-hydroxyphenyl)retinamide–regulated genes in human umbilical vein endothelial cells.** To further explore the molecular mechanisms behind 4HPR-induced changes in HUVECs, we examined early changes in cellular gene expression by oligonucleotide microarray transcriptome analyses. We used Affymetrix GeneChip Human Genome U95Av2 arrays that contain 12,500 probe sets for human transcripts that are largely well annotated. Each probe set is composed of 16 perfect match and 16 mismatch oligonucleotide 25-mers. Data were collected from three GeneChips for each condition that were hybridized with cRNA prepared from three independent experiments with HUVECs obtained from different donors (biological replicates). From the data obtained when analyzing HUVECs after 5-hour exposure to 5 \( \mu \)mol/L 4HPR, only 28 genes passed a threshold of 2-fold up-regulation or down-regulation (Table 1). Several genes of this list were picked randomly for real-time PCR validation that confirmed their modulation (Fig. 4A). We then analyzed the molecular pathways targeted that were represented in a set of 243 genes that passed a threshold of 1.4-fold up-regulation or down-regulation. Using the EASE program (26), 669 pathway annotations were available for the genes expressed in HUVECs, 27 of which were contained in the extended list of 4HPR-regulated genes. Among the 27 genes regulated by 4HPR, 5 belonged to the TGF-\( \beta \) signaling pathway (EASE score of 0.046) compared with 36 of 669 of the population of expressed genes. No other pathway yielded a score of <0.05. Consistent with this, the analysis of Gene Ontology "molecular function" showed that genes encoding for signaling molecules were significantly enriched among the 4HPR-responsive genes.

Based on these data and our particular interest in candidate genes that could explain the antiangiogenic effects of 4HPR, we focused on ligands belonging to the TGF-\( \beta \) family. We carried out real-time PCR validation for BMP-2 as well as for MIC-1, a gene also up-regulated by 4HPR although to a lesser degree.

![Fig. 2. Effect of 4HPR on growth and organization of HUVECs in the Matrigel morphogenesis assay. HUVECs spontaneously form capillary-like networks after 6 hours of incubation at 37°C. 4HPR inhibited this process as well as the growth of endothelial cells after as little as 6 hours even at the lowest concentration used.](www.aacrjournals.org)
In our experience, array data can often underestimate the extent of regulation compared with real-time PCR validation. In fact, real-time validation showed that both genes were dose-dependently and significantly ($P < 0.001$, t-test) induced after treatment with 1 or 5 μmol/L 4HPR (Fig. 4B). The effect of 4HPR was not restricted to HUVECs, as other endothelial cell lines of different origin showed similar patterns of BMP-2 and MIC-1 up-regulation (Fig. 4B). The induction of both BMP-2 and MIC-1 appeared to be a direct effect of 4HPR as mRNA for both proteins accumulated after 4HPR treatment even in the presence of a cycloheximide block of protein synthesis; further, BMP-2 and MIC-1 induction by 4HPR was reversed after 5 hours of chase (data not shown).

We then investigated whether the induced mRNA species corresponded to an induction of the encoded proteins. Increased levels of secreted BMP-2 were found after 24- and 48-hour exposure of HUVECs to 5 μmol/L 4HPR (Fig. 5A). No intracellular BMP-2 could be detected by either ELISA or Western blotting analyses (data not shown). Western blotting analysis showed an augmented cellular MIC-1 protein content, although this was not associated with increased secretion (Fig. 5B; data not shown). We therefore proceeded to functionally validate these two proteins.

Table 1. Gene regulation data derived from microarray screening of 4HPR-treated HUVECs

| Gene symbol | Gene title                                      | Fold change |
|-------------|------------------------------------------------|-------------|
| **4HPR-regulated genes**               |                        |             |
| DHRS3       | Dehydrogenase/reductase                         | 3.19        |
| SELE        | Selectin E (endothelial adhesion molecule 1)   | 3.06        |
| NRIP1       | Nuclear receptor interacting protein 1          | 2.89        |
| ATF3        | Activating transcription factor 3               | 2.85        |
| GADD153     | Growth and DNA damage – inducible transcription factor 153 | 2.80        |
| PTGS2       | Prostaglandin-endoperoxide synthase 2          | 2.74        |
| SEMA3C      | Semaphorin 3C                                   | 2.61        |
| CXCL2       | Chemokine (C-X-C motif) ligand 2                | 2.45        |
| BMP2        | Bone morphogenetic protein-2                   | 2.43        |
| CXCL3       | Chemokine (C-X-C motif) ligand 3                | 2.40        |
| IRF1        | IFN regulatory factor 1                         | 2.35        |
| HERPUD1     | Homocysteine-inducible                          | 2.28        |
| SLC7A5      | Solute carrier family 7                         | 2.27        |
| KLF7        | Kruppel-like factor 7                           | 2.25        |
| TncRNA      | Trophoblast-derived noncoding RNA               | 2.23        |
| TRIM33      | Tripartite motif-containing 33                  | 2.16        |
| DDIT4       | DNA damage – inducible transcript 4             | 2.13        |
| KLF7        | Kruppel-like factor 7                           | 2.08        |
| CCL20       | Chemokine (C-C motif) ligand 20                 | 2.03        |
| C10orf10    | Chromosome 10 open reading frame 10             | 2.03        |
| BHLHB2      | Basic helix-loop-helix domain, class B, 2      | 2.01        |
| RAI3        | Retinoic acid induced 3                         | 2.01        |
| SIAT4A      | Sialyltransferase 4A                            | 2.01        |
| TFF3        | Trefoil factor 3                                | 0.49        |
| HSPA2       | Heat shock 70-kDa protein 2                     | 0.47        |
| PPP1R3C     | Protein phosphatase 1, regulatory subunit 3C    | 0.44        |
| CD34        | CD34 antigen                                    | 0.38        |
| LDLR        | Low-density lipoprotein receptor                | 0.37        |

**NOTE:** The mean values are derived from three independent experiments and their respective analyses. Those genes that showed at least 2-fold variation (0.5-fold for down-regulated genes) in all replicates of 4HPR-treated cells compared with vehicle-treated control cells are reported.

In our experience, array data can often underestimate the extent of regulation compared with real-time PCR validation. In fact, real-time validation showed that both genes were dose-dependently and significantly ($P < 0.001$, t test) induced after treatment with 1 or 5 μmol/L 4HPR (Fig. 4B). The effect of 4HPR was not restricted to HUVECs, as other endothelial cell lines of different origin showed similar patterns of BMP-2 and MIC-1 up-regulation (Fig. 4B). The induction of both BMP-2 and MIC-1 appeared to be a direct effect of 4HPR as mRNA for both proteins accumulated after 4HPR treatment even in the presence of a cycloheximide block of protein synthesis; further, BMP-2 and MIC-1 induction by 4HPR was reversed after 5 hours of chase (data not shown).

We then investigated whether the induced mRNA species corresponded to an induction of the encoded proteins. Increased levels of secreted BMP-2 were found after 24- and 48-hour exposure of HUVECs to 5 μmol/L 4HPR (Fig. 5A). No intracellular BMP-2 could be detected by either ELISA or Western blotting analyses (data not shown). Western blotting analysis showed an augmented cellular MIC-1 protein content, although this was not associated with increased secretion (Fig. 5B; data not shown). We therefore proceeded to functionally validate these two proteins.

**Bone morphogenetic protein-2 and macrophage inhibitory cytokine-1 repress human umbilical vein endothelial cell proliferation, migration, and invasion but not network formation.** If BMP-2 and MIC-1 mediate the effects of 4HPR on endothelial cells, they would be expected to yield similar effects on HUVEC proliferation, migration, and invasion in vitro. Treatment of HUVECs with various concentrations (0-100 ng/mL) of BMP-2 and MIC-1 for 48 and 96 hours produced partial cell growth suppression, with the maximal effect observed at 100 ng/mL after 96 hours (data not shown), similar to in vitro growth suppression observed previously with 4HPR (16). Under the same conditions, neither BMP-2 nor MIC-1 were able to induce apoptosis (data not shown), again similar to that observed previously for 4HPR (16). However, like 4HPR, both BMP-2 and MIC-1 strongly inhibited HUVEC migration and invasion through Matrigel (Fig. 6A and B) in response to NIH3T3-CM ($P < 0.001$, two-tailed t test) at 50 to 100 ng/mL concentrations. Lower concentrations had no effect (data not shown).

To assess whether inhibition of HUVEC migration and invasion by 5 μmol/L 4HPR could directly be linked to BMP-2 production, we carried out a series of experiments where cells were incubated simultaneously with 4HPR and neutralizing antibodies to BMP-2. Anti-BMP-2 antibody (10 μg/mL)
essentially completely reversed the inhibitory effects of 4HPR on HUVEC migration (Fig. 6C) and invasion (data not shown). Lower antibody concentrations (0.5-5 μg/mL) showed a dose-dependent inhibition, whereas addition of irrelevant antibodies had no effect on either HUVEC migration or inhibition by 4HPR (Fig. 6C).

Because these molecules inhibited HUVEC invasion in vitro similar to 4HPR, we investigated whether they also affected the metalloprotease balance during invasion. Interestingly, supernatants from the cells in the invasion assays in the presence of 50 and 100 ng/mL BMP-2 or MIC-1 showed a marked decrease in the MMP-2 activity produced by the HUVECs (Fig. 6D). However, unlike 4HPR, the CD13/APN activity in HUVECs exposed to increasing concentrations of BMP-2 or MIC-1 remained constant (data not shown).

HUVECs were plated on Matrigel-coated plates in complete medium and treated with or without BMP-2 and MIC-1 at 50 and 100 ng/mL to study whether the two molecules influenced morphogenesis. No differences in the formation of networks structures were observed between control and treated cells (data not shown).

**Bone morphogenetic protein-2 and macrophage inhibitory cytokine-1 abrogate angiogenesis in vivo.** Because the effects of BMP-2 and MIC-1 on angiogenesis-associated endothelial cell functions observed in vitro were similar to that of 4HPR and typical of several angiogenesis inhibitory molecules, we then examined their ability to inhibit angiogenesis in vivo in the Matrigel sponge assay. Matrigel suspensions containing a cocktail (VTH) of vascular endothelial growth factor (100 ng/mL) and tumor necrosis factor-α (2 ng/mL) as angiogenic stimuli were injected s.c. in mice. The presence of VTH in the Matrigel sponges promoted a hemorrhagic vascularization of the gels within 4 days. Quantification of the extent of angiogenesis by hemoglobin content measurement showed that inclusion of BMP-2 or MIC-1 at 100 ng/mL potently and significantly reduced (BMP-2: \( P = 0.001 \), MIC-1: \( P = 0.0017 \), Mann-Whitney) the angiogenic response with respect to the positive control (Fig. 7A). The combination of

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**Fig. 4.** A, real-time PCR expression validation and comparison with microarray results. RNAs extracted from control and 4HPR-treated HUVECs were reverse transcribed into cDNA and amplified using selected primers (sequences available on request). Amplification products were revealed by SYBR Green fluorescence measurements in real time. Relative expression was assessed after normalization on glyceraldehyde-3-phosphate dehydrogenase expression data obtained from reactions run in parallel. All amplifications were done in triplicate. The logarithm of the fold change in expression data from real-time reverse transcription-PCR (RT RTPCR; grey columns) was compared with those from the microarray chips (MA; black columns). B, analysis of 4HPR-dependent induction of BMP-2 and MIC-1 in different endothelial cell lines by real-time reverse transcription-PCR. Total RNA was isolated from control cells and cells exposed for 5 hours to 1 or 5 μmol/L 4HPR as indicated. Amplification products were measured during the reaction, and mean normalized expression values were calculated by comparison with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase, amplified in parallel.
BMP-2 and MIC-1 (both at 100 ng/mL) produced an even more evident reduction of vascularization (P < 0.0001, Mann-Whitney; Fig. 7A). To confirm a role for BMP-2 in the antiangiogenic effects of 4HPR, neutralizing antibodies to BMP-2 were used. As reported previously (16), inclusion of 5 μmol/L 4HPR into the Matrigel sponges essentially abrogated angiogenesis (P < 0.001 with respect to untreated positive control). The addition of neutralizing anti-BMP-2 antibodies (15 μg/mL) to 4HPR-containing sponges reversed the angiogenic repression induced by 4HPR, producing an angiogenic response similar to the positive controls (Fig. 7B). The inclusion of irrelevant isotype-matched antibodies had no effects on either angiogenesis or 4HPR activity. The anti-BMP-2 antibodies alone did not affect angiogenesis.

**Discussion**

Recent gains in the knowledge of endothelial cell physiology and tumor angiogenesis are providing the necessary background to develop effective antiangiogenic strategies to specifically target tumor angiogenesis. Angiogenesis appears to be critical in the early stages of tumor progression, the “angiogenic switch” (27) that premalignant lesions depend on for conversion into invasive cancer. It is also increasingly evident that angiogenesis may be most effectively inhibited before the angiogenic switch, a concept known as angio prevention (28).

4HPR has been reported to inhibit the growth of early-stage prostate cancer when given from the time of tumor cell injection in a prevention protocol (17). We showed that 4HPR administration inhibited growth of established Kaposi’s sarcoma tumors in vivo in early intervention protocols (16). Inhibition of retinoblastoma tumor growth by 4HPR treatment in both prevention and intervention settings was also associated with angiogenesis inhibition (29). Further, growth inhibition of neuroblastoma biopsies in the chick chorioallantoic membrane assay by 4HPR was also associated with inhibition of angiogenesis (18). Taken together, these data indicate that 4HPR inhibits tumor growth through mechanisms involving the modulation of angiogenesis-associated growth factors and their receptors on both tumor and endothelial cells, clearly linking the antiangiogenic and antitumor activity of 4HPR.

Although 4HPR did not strongly affect HUVEC proliferation (16), it significantly inhibited endothelial cell migration and invasion toward angiogenic factors (fibroblast supernatants) and reorganization on Matrigel. 4HPR also induced a dose-dependent decrease of MMP-2 and CD13/APN activities released by HUVECs. MMPs appear to be involved in many stages of tumor progression and angiogenesis, from inducing the angiogenic switch to tumor cell invasion itself (30), and CD13/APN plays an important functional role in vasculogenesis, as APN antagonists specifically inhibited angiogenesis in chorioallantoic membranes and in the retina and suppressed tumor growth (25, 31). These protease repression activities may be partially responsible for inhibition of invasion *in vitro* and angiogenesis *in vivo*.

To further explore the molecular mechanisms behind 4HPR-induced changes in HUVECs, we examined 4HPR-induced changes after 5 hours in cellular gene expression using 12,500 gene microarrays. Gene Ontology molecular function analysis showed that genes encoding for signaling molecules were significantly enriched among the 4HPR-responsive genes. Consistent with the previous observations that 4HPR does not induce apoptosis in HUVECs (16), the Gene Ontology class “apoptosis” was not overrepresented among the 4HPR-responsive genes. We therefore focused on two members of the TGF-β family that were up-regulated in several endothelial cell lines by 4HPR: BMP-2 and MIC-1 (also known as GDF-15, PLAB, and NAG-1).

Growth factors of the TGF-β family inhibit the proliferation of epithelial, endothelial, and hematopoietic cells, but they also have the paradoxical function of promoter of tumor progression and metastasis (32). Because the 4HPR-induced changes in BMP-2 and MIC-1 expression were confirmed by real-time reverse transcription-PCR and protein analyses and occurred at 4HPR concentrations attainable in the clinic (33, 34), we further explored their role in mediating 4HPR-induced growth arrest of endothelial cells and possibly angiogenesis inhibition.

Pronounced changes take place in endothelial cells upon the application of TGF-β, including growth inhibition, increased matrix deposition, and suppression of cell-associated proteolytic...
activity (35). Relationships between these effects have shed some light on the mechanism of action of TGF-β and on its role in regulating angiogenesis. For instance, preliminary evidence has indicated that increased levels of certain matrix components may be partly responsible for the antiproliferative action of TGF-β. In addition, TGF-β and basic fibroblast growth factor have opposing effects on the cellular proteolytic balance; this may contribute to the antagonistic effect that TGF-β has on basic fibroblast growth factor–induced endothelial cell growth and possibly to the antiangiogenic effect exerted by TGF-β under certain circumstances (35).

Several reports indicated interactions of retinoids and TGF-β in the regulation of cell differentiation and proliferation (36). Retinoids, and in particular retinoic acid, regulate programmed cell death or differentiation by promoting BMP-2 and MIC-1 gene expression in different cell lines and systems (37–44). Studies have suggested that BMPs regulate vasculogenesis during embryonic development (45), but the role of BMP-2 in the induction of a blood supply in postnatal tissues is still controversial, as it has also been reported to stimulate angiogenesis in developing tumors (46) and during bone formation (47). Our results point to an antiangiogenic activity of BMP-2, as addition of purified BMP-2 protein to HUVECs resulted in growth arrest (data not shown), which was evident at 96 hours, whereas 6 hours were sufficient to inhibit migration and invasion and to decrease the activity of MMP-2 released in the medium during the assay.

We reported previously (16) that 4HPR inhibited in vivo tumor growth and angiogenesis by decreasing the secretion of vascular endothelial growth factor by tumor cells and the
expression of its receptor (vascular endothelial growth factor receptor 2) on endothelial cells. Here, we show a new mechanism through which 4HPR exerts antiangiogenic activity by up-regulating BMP-2 and MIC-1. The inclusion of BMP-2 or MIC-1 in the Matrigel in vivo angiogenesis assay confirmed the antiangiogenic activity of these two molecules. Further, function-blocking anti-BMP-2 antibodies reversed the effects of 4HPR in vitro and in vivo, clearly indicating that this molecule plays a role in transduction of key antiangiogenesis signals. However, although BMP-2 appears to be critical in mediating some processes induced by 4HPR, the retinoid clearly has activities independent of BMP-2, such as reorganization on Matrigel or repression of CD13/APN activity. This may involve other pathways, such as production of reactive oxygen species (29), independent of those effects mediated by the two cytokines. Our data suggest that BMP-2 and/or MIC-1 lie in an important pathway of 4HPR action on endothelial cells.

Biochemical studies in endothelial cells and genetic studies in mice and humans have yielded insights into the role of TGF-β, and its downstream Smad effectors, in embryonic vascular morphogenesis and in the establishment and maintenance of vascular integrity. Two distinct TGF-β signaling cascades within endothelial cells are involved, the activin receptor-like kinase 5-Smad2/3 pathway and the activin receptor-like kinase 1-Smad1/5 pathway (48), both being implicated in angiogenesis (49). In different endothelial cell lines, the expression of a constitutively active form of activin receptor-like kinase 1 inhibited cell proliferation and migration and these phenomena were associated with MIC-1 mRNA induction (49). The increase in MIC-1 expression we observed in the less angiogenic phenotype of endothelial cells exposed to 4HPR supports these observations, which are further strengthened by the data we obtained on endothelial cells in vitro with purified MIC-1. Six hours are in fact sufficient to strongly inhibit migration and invasion and to decrease the activity of MMP-2 released by the cells. Decreased cell growth takes place at later times but is already evident after 24 hours. MIC-1 is an important downstream mediator of p53 function, acting as an intercessor of cellular stress signaling and exerting antitumorigenic activities (50–52). Expression of the stress-induced transcription factor GADD153 was up-regulated in response to 4HPR in cell lines undergoing apoptosis, such as neuroblastoma and hepatoma (53, 54). From our array screening, we also found that HUVECs exposed to 4HPR showed up-regulation of two stress-related genes: GADD153 and HERPUD1 (homocysteine-inducible, endoplasmic reticulum stress-inducible) that could be directly related to 4HPR treatment or to MIC-1 induction or both of them.

In summary, we show that 4HPR represses angiogenesis by modulating endothelial cell functions, such as cell growth, organization, migration, and invasion, the latter by decreasing cell-associated protease activity. The up-regulation of TGF-β-related molecules, which have been described to directly control endothelial cell functions and tumor cell growth, may contribute to the antiangiogenic activity of 4HPR. The precise role of TGF-β in angiogenesis has proven to be confusing and context dependent; we show the novel finding that two related proteins, BMP-2 and MIC-1, mediate cell growth, migration, invasion, and angiogenesis in endothelial cells. All these combinations of activities should increase the efficacy of 4HPR therapy in tumors and provide a strong rationale for further investigation.

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