Heme oxygenase-1 promotes angiogenesis in urothelial carcinoma of the urinary bladder

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Abstract. Angiogenesis is necessary for the growth, invasion, and metastasis of solid tumors. Previous studies have shown that heme oxygenase-1 (HO-1) plays an important role in angiogenesis in both normal and cancerous cells, such as vascular endothelial cells and pancreatic cancer cells, respectively. In this study, we analyzed the role of HO-1 and other angiogenic factors in urothelial carcinoma of the bladder. Specifically, we used real-time reverse transcription polymerase chain reaction (PCR) and Western blotting to investigate the upregulation of 7 angiogenic factors, namely, HO-1, vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF)-1α, HIF-2α, cyclooxygenase-2 (COX-2), interleukin-8 (IL-8), and basic fibroblast growth factor (bFGF) under hypoxic conditions in the T24 urothelial carcinoma cell line. We also used enzyme-linked immunosorbent assay (ELISA) to measure the amount of VEGF secreted into the growth media. In addition, we administered an HO-1 inhibitor, zinc protoporphyrin IX, to mice with subcutaneous T24 tumors to assess the modulation of angiogenesis in solid tumors in vivo. We also performed immunohistochemical analyses of 23 primary bladder cancer specimens with high-grade tumors infiltrating into the stroma (pT1) for expression of HO-1, VEGF, HIF-1α, HIF-2α, COX-2, and CD31. Image analysis of CD31 staining was performed to estimate microvessel density (MVD), a measure of angiogenesis. Hypoxic conditions induced upregulation of HO-1, VEGF, HIF-1α, HIF-2α, and COX-2 in T24 cells and increased VEGF secretion, which could be suppressed by zinc protoporphyrin IX. In vivo, inhibition of HO-1 decreased tumor growth and MVD by suppressing angiogenic factors, particularly VEGF and HIF-1α. In clinical specimens of bladder cancer, high expression of HO-1 was correlated with high expression of HIF-1α (P=0.027) and high MVD (P=0.005), but not with VEGF expression (P=0.19). In conclusion, since overexpression of HO-1 promotes angiogenesis in urothelial carcinoma cells, HO-1 inhibitors could be used as novel therapeutics for urothelial carcinoma of the urinary bladder.

Introduction

In the US, urothelial carcinoma (UC) of the urinary bladder is the fourth most common malignancy in men and the ninth most common malignancy in women (1). Approximately 70% of all newly diagnosed bladder cancer cases involve non-muscle invasive primary tumors (pTa-1 or pTis), which are usually managed by transurethral surgery, intravesical chemotherapy, or Bacillus Calmette-Guerin (BCG) immunotherapy (2). Generally, patients with BCG-refractory, non-muscle, invasive, high-grade tumors or advanced stage tumors (pT2, N1, or M1) have poor prognosis. Although multidisciplinary treatments, including radical cystectomy, cisplatin-based systemic chemotherapy, and radiotherapy, are effective, some patients relapse and die of the disease (2). As a result, novel therapeutics need to be developed.

Angiogenesis is essential for the growth of solid tumors, such as bladder UC (3). In addition, neovascularization accompanies accelerated tumor growth and is strongly correlated with the invasion and metastasis of several malignancies, including bladder cancer (3-6). As a result, bladder UC is characterized by hypervascularity. The extent of vascularization and angiogenesis can be measured by tumor microvessel density (MVD), a prognostic indicator for bladder UC (4,7-10). Many types of cells secrete various endogenous angiogenic activators and inhibitors that regulate tumor angiogenesis (11). For example, bladder UC cells produce high levels of vascular endothelial growth factor (VEGF) (12), basic fibroblast growth factor (bFGF) (13), and interleukin-8 (IL-8) (14). These angiogenic factors play important roles in the promotion of angiogenesis and subsequent metastasis (15). Moreover, the expression levels of these factors correlate with tumor stage and disease outcome (6,15). As a result, it is hypothesized that inhibiting angiogenesis, either alone or in combination with conventional chemotherapeutics, will improve the outcome in bladder UC patients. Currently, anti-angiogenic agents, such as sunitinib, sorafenib, pazopanib, and bevacizumab, are being used to treat advanced UC of the bladder (6).

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in the oxidation of heme to carbon monoxide (CO), biliverdin,
and ferrous iron (Fe++) (16). HO-1 is induced by various stresses, such as oxidative stress and hypoxia-ischemia (16). HO-1 and its products are important for maintaining cellular homeostasis and have antioxidant, anti-inflammatory, and anti-apoptotic effects (17). Previous studies have demonstrated that elevated HO-1 levels affect tumorigenesis, growth, and resistance to chemo- and radiotherapy in several malignancies (16,18-21). In addition, HO-1 has a complex role in the stimulation of angiogenesis and metastasis (17,22,23). For example, in human pancreatic cancer, overexpression of HO-1 potentiates tumor aggressiveness by promoting angiogenesis and metastasis (22). Similarly, in human gliomas, HO-1 expression is correlated with macrophage infiltration and neovascularization (23).

Hypoxia, a major initiator of angiogenesis, activates transcription factors, such as hypoxia-inducible factors (HIF)-1α and HIF-2α that induce the expression of angiogenic genes (24). HIFs are members of the basic helix-loop-helix gene family. Under normoxic conditions, HIF-1α is rapidly degraded by the ubiquitin-dependent proteasome pathway. However, hypoxic conditions increase HIF-1α stability and transcription, which increases the expression of target genes involved in erythropoiesis, glycolysis, and angiogenesis (25). HIF-2α, originally called endothelial PAS protein-1 (EPAS1), shares close amino acid sequence homology and similar pharmacological and regulatory properties with HIF-1α (24,25). In addition, there is increasing evidence that cyclooxygenase-2 (COX-2) promotes tumor angiogenesis by regulating HIF-1α and VEGF, even in cases of gastric cancer (25-27). Furthermore, prostaglandin E2, a product of COX-2, is directly involved in the formation of new blood vessels (11).

Previous studies show that VEGF and HIF-1α accelerate angiogenesis by upregulating HO-1 in endothelial cells (17,28,29). However, it is unclear whether HO-1 is associated with angiogenesis and other angiogenic factors in UC of the bladder. True understanding of molecular mechanism related to angiogenesis has opened avenues for the optimal use of antiangiogenic therapy. As a result, the aim of this study was to clarify the role of HO-1 in angiogenesis in UC of the bladder. Specifically, we assessed the expression of HO-1 and its association with 6 angiogenic factors, namely, VEGF, bFGF, IL-8, HIF-1α, HIF-2α and COX-2, using a UC tumor cell line, mouse xenograft model, and clinical specimens of UC of the bladder.

## Materials and methods

**Chemical compounds.** Two hypoxia-mimetic compounds, Cobalt(II) chloride (CoCl2, 100 mM), deferoxamine mesylate (DFO), and hemin, an HO-1 inducer, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Zinc protoporphyrin IX (ZnPP), an HO-1 inhibitor, was obtained from Enzo Life Sciences (Farmingdale, NY, USA). DFO and hemin, and ZnPP were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100, 10, and 50 mM, respectively. The stock solutions were stored at -20°C before use.

**Cell culture.** The T24 UC cell line (19), which originated from a high-grade invasive bladder tumor, was maintained in RPMI-1640 growth medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (ICN Biomedicals, Aurora, OH, USA), 100 μM penicillin, and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA) in a standard humidified incubator at 37°C in an atmosphere of 5% CO2.

**RNA extraction and quantitative real-time reverse transcription PCR analysis.** T24 cells were seeded in 6-well plates at a density of 1x10^5 cells/well in growth medium and incubated for 24 h. Then they were treated with the indicated concentrations of CoCl2, DFO, and hemin. After incubating the plates for 48 h, total RNA was extracted by using the RNeasy mini kit (Qiagen, Hilden, Germany). Total RNA (1 μg) was reverse transcribed in a final volume of 20 μl with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed with a StepOnePlus™ Real-Time PCR system (Applied Biosystems) in a 20 μl reaction volume containing 1 μl of the first-strand cDNA, 0.2 μM of each primer, and 10 μl of Power SYBR Green Master mix (Applied Biosystems) under the following conditions: 95°C for 10 min; 40 cycles of 95°C for 15 sec, 55°C for 15 sec, and 60°C for 1 min. Subsequently, a melting curve analysis was used to check the specificity of amplification. The mRNA expression level was normalized to the expression of the β-actin gene, ACTB. The primers used in this study were: ACTB forward CTGGA CGGTGAAAGCTGACA and reverse CGGCCACATTTGTG AACTTTG, HO-1 forward GTGTGATAGAAGAGGCC AAGAC and reverse GCAGAAATCTTGCACTTTGTTG, COX-2 forward TGCATTCTTGGCCACAGCT and reverse AAAGGCCAGTTTAGCTGTG, HIF2A forward GGA GAACAGCAAGAGCGT and reverse GGGACGAGGT AGGACTCAA, IL8 forward GTGCAGTTTGGCCA AGAGT and reverse CCTCTGACCCAGCTTTC, VEGF forward CCGCAGACGTGGAAGTGCTCT and reverse CGCGTGTCTGAATCGCTT and reverse CGCGTGTCTGACATCTGAGTA, and bFGF forward CCGGACCGCGGAGGATC and reverse CACAT TTAGAAGCCGTAATCT.

**Western blot analysis.** Since HIF-1α regulates post-transcription (30,31), Western blot analysis was used to detect upregulation of HIF-1α. After incubating the cells in 100 μM of CoCl2, DFO, and hemin for 48 h, total protein was extracted. Protein extraction, measurement of protein concentration, and immunoblotting were performed as previously described (19). The primary antibodies, rabbit polyclonal anti-HIF-1α (1:100 dilution, Upstate Biotechnology, Lake Placid, NY, USA) and anti-actin mouse monoclonal antibody (1:3000 dilution, Sigma-Aldrich), which was used as an internal loading control, were incubated at 4°C overnight. Finally, the bound secondary antibody (1:10000 dilution for 1 h) was detected by using enhanced chemiluminescence (ECL) in the Western Blotting Detection system (GE Healthcare, Little Chalfont, UK).

**Measurement of secreted VEGF in vitro.** T24 cells were seeded in a 96-well plate at a density of 5x10^3 cells per well. After preincubation for 16 h, the cells were treated with or without 10 μM ZnPP, 100 μM CoCl2, or 100 μM of DFO for 24 h. The amount of secreted VEGF in the culture supernatant was determined by using the Human VEGF Assay kit (R&D Systems, Minneapolis, MN, USA).
treatment group, ZnPP (12.5 mg·kg⁻¹) was injected intra-
muscularly into each mouse. When the tumors reached 5 mm in diameter, the animals were divided randomly into a control group (n=4) or ZnPP treatment group (n=8). Treatment was initiated on day 0.

**Mouse xenograft model.** Female athymic BALB/c nu/nu mice (8 weeks old) were maintained under pathogen-free conditions and provided with sterile food and water. T24 cells (2x10⁶) in 100 μl RPMI-1640 medium and 100 μl of Matrigel (Becton Dickson, Bedford, MA), were injected subcutaneously into the flank of each mouse. When the tumors reached 5 mm in diameter, the animals were divided randomly into a control group (n=4) or ZnPP treatment group (n=8). Treatment was initiated on day 0. ZnPP was dissolved in DMSO at a concentration of 100 mg/ml, and then diluted in phosphate-buffered saline (PBS). In the treatment group, ZnPP (12.5 mg·kg⁻¹) was injected intraperitoneally into the mice once per week for 3 weeks. In the control group, mice only received the injection vehicle on the same schedule. The diameter of tumors were measured on day 21 with electronic calipers, and then their volumes were calculated by using the formula: volume (mm³) = (width x length)²/2. Afterwards, the mice were sacrificed and the tumors were resected.

**Immunohistochemical analysis of xenograft tumors.** Tumors were fixed in 10% formaldehyde solution and embedded in paraffin. Immunohistochemical (IHC) staining was performed as previously described (20). The primary antibodies and incubation conditions were as follows: 1) mouse monoclonal anti-HO-1 (BD Transduction Laboratories, San Diego, CA, USA), 1:100 dilution, 4˚C overnight; 2) rabbit polyclonal anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:50 dilution, 4˚C overnight; 3) rabbit polyclonal anti-HIF-1α (Upstate Biotechnology, Lake Placid, NY, USA), 1:100 dilution, 37˚C for 1 h; 4) rabbit polyclonal anti-HIF-2α (Novus Biologicals, Littleton, CO, USA), 1:100 dilution, 37˚C for 1 h; 5) mouse monoclonal anti-COX-2 (Cayman Chemical, Ann Arbor, MI, USA), 1:100 dilution, 4˚C overnight; and 6) rabbit polyclonal anti-PECAM1 (CD31) (Santa Cruz Biotechnology), 1:500 dilution, 4˚C overnight. For verification purposes, some tumor cells were counterstained with hematoxylin and eosin (H&E).

**Microvessel density analysis.** Microvessel density (MVD) of xenograft tumors and clinical specimens of UC was determined by using the method of Weidner et al (5). The slides that were stained with anti-CD31 antibody were scanned at low magnification (x40) to identify the 5 areas with the highest MVD. Then, the number of stained blood vessels in a high magnification field (x200) in each of these areas was estimated. Subsequently, the MVD score was calculated as the mean of these 5 quantities.

**Patients and tissue samples.** The Nara Medical University (Nara, Japan) institutional review board approved this study and all participants provided informed consent. Formalin-fixed and paraffin-embedded tissue blocks were made from operative specimens of UCs. Standard H&E slides were analyzed according to the criteria of the 2004 World Health Organization (WHO) and TNM cancer staging system (32,33). Specimens were obtained from 23 patients pathologically diagnosed with T1 and high-grade (pT1/HG) UC who underwent transurethral resection between January 2000 and December 2005 at the Nara Medical University Hospital. The paraffin blocks were cut into 3-μm-thick sections and mounted onto aminoalkyl-silane-coated glass slides. IHC staining for HO-1, VEGF, HIF-1α, HIF-2α and COX-2 was performed in a similar manner as described above for xenograft tumors.

The HO-1 staining was evaluated by a semiquantitative system that graded IHC patterns with scores of the extent and intensity of staining ranging from 0 to 9 (34). Specifically, the extent of staining was scored on a scale from 0 to 3, according to the following criteria: 0, no detectable staining; 1, <10% scattered cells; 2, 10-50% stained cells; 3, >50% homogeneously stained cells. Similarly, the intensity of staining was scored according to the following criteria: 0, no detectable staining; 1, weakly stained; 2, moderately stained; 3, strongly stained cytoplasm or nuclei. Final scores were calculated by multiplying these 2 scores.

In the evaluation of VEGF, HIF-1α, HIF-2α and COX-2, all reactive nuclei or cytoplasm were considered positive regardless of their intensity. The threshold percentages that were used to distinguish between low and high expression levels were established prior to statistical analysis of the data. Specifically, these percentages were defined as 10% for VEGF (35), 50% for HIF-1α (24), 50% for HIF-2α (24), and 10% for COX-2 (27). IHC evaluations were performed independently by two investigators in this study (M.M. and T.A.). Any discrepancies between their assessments were resolved by re-assessing the slides in question in a combined batch without knowledge of the previous scores.

**Statistical analysis.** PRISM software version 4.00 (GraphPad Software, San Diego, CA) was used for statistical analyses. P<0.05 were considered to be statistically significant.

**Results**

**Hypoxic conditions induce expression of HO-1 and angiogenic factors in UC cells.** We used real-time RT-PCR and Western blot analyses to determine correlations among the expression of HO-1 and 6 angiogenic or hypoxic factors, namely, HIF-1α, HIF-2α, COX-2, IL-8, bFGF, and VEGF. In this experiment, 2 hypoxia-mimetic agents, CoCl₂ and DFO, were used to simulate hypoxic conditions in the T24 cell line in vitro. After 48 h of treatment, the mRNA expression of HO-1, HIF-2α, COX-2, and VEGF (Fig. 1A) and the protein expression of HIF-1α (Fig. 1B) increased significantly. Since no significant differences were observed in the expression of IL-8 or bFGF (data not shown), these factors were excluded in the IHC analysis. Overexpression of HO-1 by hemin was not correlated with the upregulation of any angiogenic factors.

**Inhibition of HO-1 decreases the secretion of VEGF in vitro.** VEGF, an important angiogenic factor, is modulated by a positive feedback loop activated by HO-1 (17). We investigated whether inhibition of HO-1 suppressed the secretion of VEGF by UC cells into the growth medium (Fig. 2). CoCl₂ and DFO stimulated VEGF secretion ~6- and 16-fold,
respectively. ZnPP effectively suppressed VEGF secretion in the presence of these hypoxia-mimetic agents. These results demonstrated that the upregulation of HO-1 under hypoxic conditions was correlated with the synthesis and secretion of VEGF by UC cells.

**Inhibition of HO-1 decreases tumor growth by suppressing angiogenesis in vivo.** Previously, it was shown that ZnPP directly inhibits cellular proliferation (20). To determine whether inhibition of HO-1 is involved in the antiangiogenic effect of ZnPP in vivo, xenograft mice with T24 cells were prepared and treated as described in Materials and methods. On day 21, the mean tumor volumes (mean ± SD) of the control and treatment groups were 356±111 mm$^3$ and 202±74 mm$^3$, respectively (P=0.026) (Fig. 3A).

Next, MVD and the expression of angiogenic factors in the tumors resected from these mice were analyzed by immunohistochemical staining (Fig. 3B-D). MVD was significantly lower in the tumors treated with ZnPP (Fig. 3B). The area with the strongest intratumoral expression of

![](https://653-660.qxd 19/1/2011 10:08 Ù"41 Æ1.656)
angiogenic factors is shown in Fig. 3D. Immunostaining of VEGF and HIF-1α was observed in the nuclei and cytoplasm of tumor cells. HIF-2α and COX-2 were expressed mainly in the cytoplasm of tumor cells. The expression levels of VEGF and HIF-1α in the ZnPP-treated tumor cells were significantly lower than those in the control cells. There was a slight difference between the expression levels of HIF-2α and COX-2 in the treated and untreated tumors. These results suggested that HO-1 may be associated with the stabilization and activation of HIF-1α, which would upregulate VEGF.

Overexpression of HO-1 correlates with angiogenesis in pT1/HG human bladder cancer specimens. To strengthen the association between HO-1 expression and angiogenesis in UC, we performed IHC analysis of clinical specimens of pT1/HG human bladder UCs. The HO-1 expression level was assessed semiquantitatively according to the reactivity (Fig. 4A). Since the mean score of HO-1 expression was 2.78±2.58, we defined a score of 3 as the threshold for distinguishing between low and high expression levels. Representative slides of other angiogenic markers with high and low expression levels are shown in Fig. 4B. Although the IHC expression patterns of VEGF, HIF-2α and COX-2 in the clinical specimens were similar to that in the xenograft tumors (Figs. 3D and 4B), there was a significant difference in the HIF-1α expression level of these two sources of tumors. Specifically, HIF-1α expression was observed predominantly in the nuclei of tumor cells in the clinical specimens, unlike its expression in both the nuclei and cytoplasm of xenograft tumors. High expression levels of HO-1 were correlated with high expression levels of HIF-1α (P=0.027) and high MVD scores (P=0.005), but not with VEGF expression levels (P=0.19) (Table I).

Discussion

Many studies have demonstrated that oncogene and oncoprotein activation plays a critical role in carcinogenesis and tumor development. Both expansion and infiltration into the stroma expose tumor cells to hypoxic conditions that promote malignant progression by inducing angiogenic factors and promoting angiogenesis. Although there is abundant evidence that HO-1 is involved in angiogenesis (17,22,23,29,30), few studies have analyzed its expression in clinical specimens.
This information is needed to elucidate the importance of HO-1 in angiogenesis. In this study, in vitro and in vivo experiments were conducted and clinical specimens of bladder UC were analyzed to determine the role of HO-1 in angiogenesis and its association with 6 angiogenic factors, namely, VEGF, bFGF, IL-8, HIF-1α, HIF-2α, and COX-2.

Treatment of T24 cells with hypoxia-mimetic agents induced expression of HO-1 and upregulated other angiogenic factors, such as HIF-1α, HIF-2α, VEGF, and COX-2 (Fig. 1A and B). However, overexpression of HO-1 by hemin did not upregulate these factors (Fig. 1A). These findings suggest that HO-1 is an angiogenic cofactor under hypoxic conditions but not normoxic conditions. This hypothesis is supported by the results of the VEGF ELISA experiments, which showed that ZnPP decreased secretion of VEGF only under hypoxic conditions (Fig. 2). IL-8, bFGF, and VEGF are important angiogenic cytokines and prognostic indicators of UC of the bladder (6,12,13). For example, in an orthotopic mouse model of human bladder UC cells, increased IL-8 and bFGF expression is associated with increased tumorigenicity and production of spontaneous lymph node metastases (36). In addition, Rogala et al showed that absorption of IL-8 by bladder cancer tissue did not reduce angiogenesis (37). In this study, hypoxia and upregulation of HO-1 were less strongly correlated with IL-8 and bFGF than with HIF-1α, HIF-2α, VEGF, and COX-2.

Table I. Correlation between expression of HO-1, angiogenic factors and microvessel density in human urothelial carcinoma of the bladder.

<table>
<thead>
<tr>
<th>Angiogenic factors</th>
<th>HO-1</th>
<th>P-value</th>
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<tbody>
<tr>
<td><strong>Total</strong></td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>10</td>
<td>6</td>
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<tr>
<td>L</td>
<td>2</td>
<td>5</td>
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<tr>
<td>HIF-1α</td>
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<tr>
<td>H</td>
<td>11</td>
<td>5</td>
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<tr>
<td>L</td>
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<tr>
<td>HIF-2α</td>
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<td>COX-2</td>
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<td>H</td>
<td>9</td>
<td>7</td>
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<td>L</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>MVD</td>
<td>39.8±9.1</td>
<td>29.1±7.3</td>
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*aFisher’s exact test; *Mann-Whitney U test.
HIF-1α and HIF-2α are closely related transcription factors that activate transcription of target genes, such as VEGF, in response to hypoxia. Previous studies show that HIF-1α and HIF-2α expression in non-muscle invasive bladder cancer may predict intravesical recurrence or progression to muscle invasive cancer (24). These transcription factors may also play important roles in the hypoxia response pathway, tumor progression, and vascularization in bladder cancer (24,38,39).

In this study, significantly lower MVD, VEGF expression, and HIF-1α expression were observed in the resected tumors treated with ZnPP (Fig. 3C). In contrast, HIF-2α expression was only slightly decreased (Fig. 3C). Jones et al demonstrated that the expression of HIF-1α and HIF-2α is higher in bladder tumors than in normal bladder tissues (38). However, in muscle invasive tumors, HIF-1α expression is more strongly correlated with VEGF expression than HIF-2α expression (38). The T24 cell line used in this study originated from an undifferentiated invasive bladder tumor. In addition, the clinical specimens of UC tumors were high-grade tumors that had infiltrated into the stroma. As a result, our results are consistent with those of Jones et al (38).

Overexpression of HO-1 in coronary microvascular endothelial cells (ECs) increases EC proliferation and formation of capillary-like structures in a 2-dimensional Matrigel assay (31). Antibodies, may be promising antiangiogenic agents. Sense oligonucleotides, small interfering RNA, and neutralizing therapeutics that inhibit HO-1 activity, such as ZnPP, anti-HIF-1α, and anti-HIF-2α, are closely related transcription factors that can specifically modulate these pathways and may be beneficial to patients with advanced stages of UC. In particular, the role of the HIF-1α/VEGF/HO-1 pathway in angiogenesis and metastasis of bladder cancer needs to be investigated further. This study suggests that therapeutics that inhibit HO-1 activity, such as ZnPP, anti-sense oligonucleotides, small interfering RNA, and neutralizing antibodies, may be promising antiangiogenic agents.

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