Hypoxia-induced, Perinecrotic Expression of Endothelial Per-ARNT-Sim Domain Protein-1/Hypoxia-inducible Factor-2α Correlates with Tumor Progression, Vascularization, and Focal Macrophage Infiltration in Bladder Cancer

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ABSTRACT

Endothelial Per-ARNT-Sim (PAS) domain protein-1 (EPAS-1)/hypoxia-inducible factor-2α (HIF-2α) is a member of the basic helix-loop-helix/PAS domain protein family and is considered to be an endothelial-specific, hypoxia-inducible transcription factor. Because hypoxia is a fundamental element of tumor biology determining clinical outcome, we performed an immunohistochemical study of EPAS-1 expression in a cohort of bladder cancer cases and assessed the possible correlation of EPAS-1 expression with tumor hypoxia and growth. In the 67 cases (37 radical cystectomy and 30 transurethral resection) studied, overexpression of EPAS-1/HIF-2α protein was not found in cancer cells or in normal tissues but was mostly found in stroma around cancer cells, and strong positive staining was noted in perinecrotic regions. The perinecrotic/tumorous expression of EPAS-1/HIF-2α was correlated statistically with higher histological grade (P < 0.001), advanced pathological T stage (P < 0.001), and presence of necrosis (P < 0.001). A parallel immunohistochemical analysis of a marker gene of vascular endothelial growth factor demonstrated its positive correlation with tumor grade, stage, and EPAS-1/HIF-2α overexpression, supporting the correlation of EPAS-1/HIF-2α upregulation with tumor angiogenesis. To further clarify the relationship between hypoxia and vascularity in the perinecrotic/tumorous area with EPAS-1/HIF-2α expression, tissue microvessel density (MVD) was assessed. No significant correlation (P = 0.442) was found between EPAS-1/HIF-2α expression and MVD if the 67 tumors of different stages were all included. However, EPAS-1/HIF-2α-positive cases had lower MVD than EPAS-1/HIF-2α-negative cases (P = 0.001) if only invasive cancer cases were analyzed. In addition, in all EPAS-1/HIF-2α-positive staining cases, EPAS-1/HIF-2α-positive foci had lower MVD than EPAS-1/HIF-2α-negative foci (P < 0.001). Finally, using serial sections, the location of EPAS-1/HIF 2α expression was identified mainly in tumor-associated macrophage (TAM) as well as in some fibroblast cells. Focal TAM infiltration was identified at a higher level in EPAS-1-positive cases than EPAS-1-negative cases (P < 0.001). This is the first clinical report suggesting that hypoxia-induced, perinecrotic EPAS-1/HIF-2α expression is correlated with tumor progression and angiogenesis at higher grade and stage through focal TAM infiltration in invasive bladder cancer.

INTRODUCTION

EPAS-1/HIF-2α is a member of the basic helix-loop-helix PAS domain protein family, which is comprised of a number of transcriptional activators important in embryonic vascular development (1, 2) and in the acquisition of specific types of memory (neuronal PAS domain protein 2; Ref. 3). EPAS-1/HIF-2α is also one of the hypoxia-inducible transcription factors, which are known to induce a cascade of physiological responses, including genes involved in hemtopoiesis (erythropoietin), angiogenesis, vasomotor (VEGF, NO synthases, endothelins, etc.), energy metabolism (glyco-lytic enzymes), catecholamine synthesis (tyrosine hydroxy-
MATERIALS AND METHODS

Patient Selection and Tissue Sample Collection. Radical cystectomy specimens from 41 consecutive patients with invasive bladder cancer and TUR specimens from 44 consecutive newly diagnosed patients with clinically superficial bladder cancer at the London Health Sciences Center, Ontario, were obtained. All surgical procedures were performed by one surgeon (J. L. C). Of the 41 patients with invasive bladder cancer, 6 had been treated with chemotherapy and/or radiotherapy before cystectomy. Four TUR cases revealed invasive cancer, and these patients underwent radical cystectomy soon after TUR without other intervening neoadjuvant therapy. Two of the 4 patients had no residual tumor in their cystectomy specimens, and thus, only the TUR specimens were included for our study and were considered T2 in the TUR group. The other 2 cystectomy cases were included in the cystectomy group. Two other cystectomy cases were pT0 likely because of neoadjuvant therapy, and 12 TUR cases had insufficient volume of tumor and stroma in paraffin blocks; thus, these cases were excluded from our study. In total, 67 cases (37 cystectomy and 30 TUR) were included for our study and were considered T2 in the TUR group.

Pathological T stage

<table>
<thead>
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<th>Pathological T stage</th>
<th>Cystectomy</th>
<th>TUR-BT</th>
<th>Total</th>
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<td>14</td>
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<tr>
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<tr>
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<td>4</td>
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</tr>
<tr>
<td>pT4</td>
<td>7</td>
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</table>

TUR-BT, transurethral resection of bladder tumor.

A mouse Mab anti-EPAS-1/HIF-2α (190b) and rabbit polyclonal anti-EPAS-1/HIF-2α antibody (PM8) were generated and characterized as reported previously (13). IHC was performed by the immuno-peroxidase technique. Briefly, deparaffinized and rehydrated blocks were obtained from the Department of Pathology. Tissue blocks were cut at 5-μm thickness and mounted on Superfrost Plus slide glasses (Fisher, Toronto, Ontario, Canada) for immunohistochemical analysis. The paraffin block that showed the most invasive tumor was selected from each case.

IHC. A mouse Mab anti-EPAS-1/HIF-2α (190b) and rabbit polyclonal anti-EPAS-1/HIF-2α antibody (PM8) were generated and characterized as reported previously (13). IHC was performed by the immuno-peroxidase technique. Briefly, deparaffinized and rehydrated sections were treated with 3% hydrogen peroxide in methanol for 15 min at room temperature to block endogenous peroxidase activity. Antigen retrieval was achieved by autoclaving in 10 mM citrate buffer (pH 6.0) for 5 min at 121°C. After blocking with 10%
goat serum in PBS, sections were incubated with the first antibody, and reaction was carried out at 4°C overnight. All first antibodies used for this study were tested for optimal dilutions, and a moderate dilution was determined for the best differentiation of tumor samples. We used 1:400-diluted EPAS-1 Mab (1.125 μg/ml) and 1:2000-diluted EPAS-1 Pab. Dilution of Mab and Pab against VEGF was 1:25 and 1:200 separately. Dilution of the Mabs CD68 (KP1) and CD31 (JC70; MediCorp, Montreal, Quebec, Canada) was at 1:800 and 1:100 separately. The avidin-biotin complex (StreptABC complex kit; DAKO, Mississauga, Ontario, Canada) technique and visualization with 3,3-diaminobenzidine/hydrogen peroxide were used to detect antigen-antibody binding. Negative controls were included by replacement of the primary antibody with PBS. All IHC slides were counterstained by hematoxylin.

ISH. A 159-bp restriction fragment (EcoRI-PsiI) of human EPAS-1 full-length cDNA clone (Ref. 1; a kind gift from Dr. S. L. McKnight) was inserted into pBluescript (pBS KS; Stratagene, La Jolla, CA) vector digested with the same enzymes. This fragment is downstream from the NH2 terminus PAS domain, which is a unique sequence for EPAS-1/HIF-2α in the HIF family (8, 20). The inserted fragment in the recombinant plasmid (201) was confirmed by complete DNA sequencing. A single-stranded, antisense RNA probe was generated by T7 RNA polymerase (Promega, Madison, WI) on EcoRI-digested and PstI-linearized recombinant plasmid and labeled with 35S-UTP. The control sense RNA probe was synthesized similarly by using PsiI digestion and T3 RNA polymerase (Promega). A standard ISH protocol was followed. In brief, clinically fixed sections of surgical specimens were deparaffinized and treated with 10 μg/ml proteinase K, 100 mM Tris, and 50 mM EDTA (pH 8.0). Slides were dehydrated; prehydrized in a buffer containing 50% form M EDTA (pH 8.0), and Target retrieval solution (DAKO). VEGF 121 ) of VEGF were used for identification and evaluation. TAM expression and VEGF, MVD, or ..

Characterization of EPAS-1/HIF-2α protein and mRNA in Bladder Cancer. Because the two antibodies against recombinant EPAS-1/HIF-2α (190b and PM8) have been characterized in a number of human normal and cancer tissues previously (13), bladder cancer samples were first tested with a Mab (190b). There was no expression of EPAS-1/HIF-2α in cancer cells in all cases (Fig. 1, a and b). However, positive staining was seen in the cytoplasm in stroma cells only around cancer cells, and more positive stains were seen near tumor and necrosis regions (Fig. 1, a and b), whereas no positive staining was seen in normal regions (Fig. 1c). To confirm the specificity of EPAS-1/HIF-2α Mab (Fig. 1a), we performed parallel immunostaining against EPAS-1/HIF-2α using a Pab that recognizes different epitopes from the Mab (13). The same staining pattern in the cytoplasm was seen between EPAS-1/HIF-2α monoclonal (Fig. 1a) and Pabs (Fig. 1b). To further confirm the perinecrotic/tumorous expression of EPAS-1/HIF-2α, single-strand 35S-UTP-labeled antisense RNA probes were

RESULTS

Validation of VEGF expression in this study. For antigen retrieval pretreatment and for optimal resolution between various stages and grades of bladder cancer, four different buffers were tested: 10 mM citrate (pH 6.0), 10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), and Target retrieval solution (DAKO). Only the first pretreatment buffer [10 mM citrate (pH 6.0)] was adopted for this study. In brief, paraffin-fixed slides were autoclaved for 7 min in the pretreatment buffer before deparaffinization and rehydration. Evaluation of the staining was semiquantitatively graded based on scores determined by intensity distribution as: strong + + + (dark brown), moderate + + (brown), weak + (light brown), or negative − (no staining). The score was determined independently by at least three of four authors (T. O., P. G. J., M. Y. G., and M. M.) independently after the blind analysis principle.

Microvessel and Focal Macrophage Detection and Quantification. Microvessel quantification was performed according to Bossi et al. (21) with some modifications. Briefly, slides were first observed at low power magnification (×100) to identify areas with the highest density of microvessels. In each case, the two most vascularized areas were selected, and microvessels of these areas were counted at high power magnification (×400). Any brown stained endothelial cell that has clearly separated from adjacent microvessels, tumor cells, and other connective tissue elements was considered to be a single countable microvessel. The presence of a vessel lumen was not required to verify the structure as a vessel. Quantification based on the blind analysis was performed independently by at least two of four authors (T. O., P. G. J., M. Y. G., and M. M.). The average of four scores for each case was taken as the MVD. For focal macrophage quantification, the three areas of highest concentration of CD68 stainings were selected, and the number of positively stained cells in each case was counted. The average number of stained cells in the three “hotspot” areas was considered as the Mql.

Statistical Analysis. A computer software Sigma Stat 2.0 program was used for statistical analysis. χ2 test was used to assess the relationship between EPAS-1/HIF-2α, VEGF, MVD, and TAM expression versus histological grade, pathological stage, and existence of necrosis. One-way ANOVA or Student’s t test was used to assess the correlation between EPAS-1/HIF-2α expression and VEGF, MVD, or Mql. P < 0.05 was considered to be statistically significant. Two non-TCC cases (squamous cell carcinoma and small cell carcinoma) were excluded from statistical analysis.

Characterization of EPAS-1/HIF-2α Protein and mRNA in Bladder Cancer. Because the two antibodies against recombinant EPAS-1/HIF-2α (190b and PM8) have been characterized in a number of human normal and cancer tissues previously (13), bladder cancer samples were first tested with a Mab (190b). There was no expression of EPAS-1/HIF-2α in cancer cells in all cases (Fig. 1, a and b). However, positive staining was seen in the cytoplasm in stroma cells only around cancer cells, and more positive stains were seen near tumor and necrosis regions (Fig. 1, a and b), whereas no positive staining was seen in normal regions (Fig. 1c). To confirm the specificity of EPAS-1/HIF-2α Mab (Fig. 1a), we performed parallel immunostaining against EPAS-1/HIF-2α using a Pab that recognizes different epitopes from the Mab (13). The same staining pattern in the cytoplasm was seen between EPAS-1/HIF-2α monoclonal (Fig. 1a) and Pabs (Fig. 1b). To further confirm the perinecrotic/tumorous expression of EPAS-1/HIF-2α, single-strand 35S-UTP-labeled antisense RNA probes were

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Fig. 1  Characterization of EPAS-1/HIF-2α expression in human bladder tumor tissues by IHC and ISH techniques.  

(a) EPAS-1/HIF-2α-positive staining (arrows) in perinecrotic region (N) near tumor foci (T) by IHC with a Mab against EPAS-1/HIF-1α in a bladder cancer sample (TCC grade 3, pT4).  

(b) serial sections of a for immunostaining by a Pab against EPAS-1/HIF-2α, showing colocalization of EPAS-1/HIF-2α-positive stainings by two kinds of antibodies (×160).  

(c) control: normal tissue showing no brown staining signal (×100).  

(d–e) detection of EPAS-1/HIF-2α mRNA by ISH using 35S-UTP-labeled, single-strand antisense RNA probe in vitro transcribed from a recombinant plasmid (201) in an invasive bladder tumor sample (TCC grade 3, pT4).  

(d) bright field view;  

(e) dark field view.  

At least two tumor foci (T) were shown in the field, and positive hybridization signals (arrows) were present only in the cytoplasm of stroma cells palisading tumor nodules.  

(f) sense strand RNA probe was used as a negative control shown by dark field illumination, which, in contrast, showed randomly spreading signals (×160).  

(g–i) immunostaining for EPAS-1/HIF-2α showing examples of ++ (g), + (h), and − (i; ×200).

Fig. 2  Parallel studies of VEGF protein expression (a and b) and MVD (c–f) in bladder cancer samples, along with EPAS-1/HIF-2α immunostaining.  

A Mab against VEGF was used for immunostaining of slides from bladder cancer patients:  

(a) TCC grade 3, pT3b showing strong staining (++) in the cytoplasm of tumor cells; and (b) TCC grade 1, pTa showing weak staining (+; ×160).  

In c–f, analysis of MVD using serial sections and immunostaining was performed with two Mabs for EPAS-1/HIF-2α (c and e) and CD31 (an endothelial cell marker; d and f).  

In two sets of serial slides showing EPAS-1/HIF-2α-positive (++) case (c) and -negative (−) case (e), there was no microvessel around EPAS-1/HIF-2α-positive case (d) and abundant microvessels (high MVD) seen in EPAS-1/HIF-2α-negative case (f; ×160).
Correlations between EPAS-1/HIF-2α Expression and Histological Grade, Pathological T Stage, and Existence of Necrosis. To investigate the correlation between EPAS-1/HIF-2α expression with hypoxia and tumor progression (higher tumor grades or stages), we evaluated EPAS-1/HIF-2α immunoreactive reaction in IHC as follows: stain in multiple areas as +++, stain only in focal area (less than two fields in ×100 magnification) as +, and the cases with no positive stain as − (Fig. 1, g–i). Thirty-three of 67 cases (49.3%) were positive, and 34 cases (50.7%) were negative for EPAS-1/HIF-2α. In the 33 EPAS-1/HIF-2α-positive cases, 5 were TUR, and 28 were cystectomy cases. No grade 1 lessons were positive, and 21.1% of grade 2 was positive, whereas 73.5% of the cases of TCC grade 3 was positive. All non-TCC cases were also positive. Positive staining was seen in 13.8% of the superficial (≦pT1) and 76.3% of the invasive (≧pT2) cancer cases (Table 2). EPAS-1/HIF-2α positivity correlated with high histological grade (P < 0.001) and advanced pathological T stage (P < 0.001). To investigate the relationship between EPAS-1/HIF-2α expression and hypoxia, we compared EPAS-1/HIF-2α expression with presence of necrosis. As shown in Table 2, 25 of 31 EPAS-1/HIF-2α-positive cases (80.6%) had necrosis, and 6 of 34 EPAS-1/HIF-2α-negative cases (17.6%) had necrosis, respectively. EPAS-1/HIF-2α positivity correlated with existence of necrosis (P < 0.001). In addition, existence of necrosis correlated with higher histological grade (P < 0.001) and advanced pathological T stage (P = 0.009). Thus, statistical data confirm that EPAS-1/HIF-2α overexpression is perinecrotic, palisading around tumor-containing areas.

VEGF Expression Is Associated with Tumor Progression and EPAS-1/HIF-2α Overexpression in Bladder Tumors. To further confirm that EPAS-1/HIF-2α in bladder cancer tissues also correlates with angiogenesis, a parallel IHC study of VEGF was performed on these 67 cases, in which VEGF expression is assessed as a major marker for angiogenesis. A Mab against isoform VEGF121 (Ab3) was first tested. Positive signals were found mostly in the cytoplasm of tumor foci (Fig. 2 a). Next, a Pab (Ab1) against isoform VEGF165 was tested, and the same pattern of positive signals in tumor foci was confirmed (data not shown). Both of these two antibodies were tested in all 67 cases, and results were compared. VEGF expression in tumors had strong correlation with histological grade (P = 0.001) and pathological stage (P = 0.001). Similar correlation with tumor grade (P = 0.007) and stage (P = 0.048) were derived with the Pab. Fig. 2, a and b showed immunostaining of VEGF signals in tumors at various grades and stages. As shown in Fig. 3, overexpression of VEGF in higher grade and stage tumors was found to correlate positively with EPAS-1/HIF-2α expression, and the numbers of cases of EPAS-1/HIF-2α + versus EPAS-1/HIF-2α − were significantly different: P = 0.027 (for Mab) and P = 0.036 (for Pab), respectively.

Spatial MVD Is Inversely Correlated with EPAS-1/HIF-2α Overexpression in Necrotic Area Palisading Tumor-Containing Areas. To study the relationship among EPAS-1/HIF-2α expression, hypoxia, and vascularization, we investigated the distribution of MVD in areas of EPAS-1/HIF-2α-positive cases. The same location of EPAS-1/HIF-2α mRNA expression in palisading tumor regions is shown in Fig. 1, d and e. ISH-positive signal was identified only in those bladder cancer cases (n = 10) with EPAS-1/HIF-2α IHC-positive expression, whereas in EPAS-1/HIF-2α IHC-negative cases (n = 12), no ISH-positive signal was found. Control experiments with sense probe generated only nonspecific background signals (Fig. 1f).

### Table 2 Correlation between EPAS-1/HIF-2α expression and histological grade, pathological T stage, or existence of necrosis

<table>
<thead>
<tr>
<th>EPAS-1/HIF-2α expression (No. of cases)</th>
<th>A.</th>
<th>B.</th>
</tr>
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<tr>
<td><strong>Histological grade</strong></td>
<td></td>
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</tr>
<tr>
<td>TCC grade 1</td>
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<tr>
<td>Grade 2</td>
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</tr>
<tr>
<td>Grade 3</td>
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<tr>
<td>Sarcomatoid TCC</td>
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</tr>
<tr>
<td>Small cell cancer</td>
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<td>1</td>
</tr>
<tr>
<td>Total</td>
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<td>13</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Total</td>
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<td>13</td>
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</table>

| Existence of necrosis (no. of cases) |
|-------------------------------------|---|
| A.                                  | 28 |
| +                                   | 2  |
| ++                                  | 4  |
| Total                               | 34 |
| B.                                  | 6  |
| Total                               | 33 |

- χ² test was used to analyze the correlation between EPAS-1/HIF-2α expression and histological grade or pathological T stage and between existence of necrosis and EPAS-1/HIF-2α expression, histological grade, or pathological T stage.

Synthesized and hybridized in situ in invasive bladder cancer samples. The same location of EPAS-1/HIF-2α mRNA expression in palisading tumor regions is shown in Fig. 1, d and e. ISH-positive signal was identified only in those bladder cancer cases (n = 10) with EPAS-1/HIF-2α IHC-positive expression, whereas in EPAS-1/HIF-2α IHC-negative cases (n = 12), no ISH-positive signal was found. Control experiments with sense probe generated only nonspecific background signals (Fig. 1f).
HIF-2α expression. MVD was measured by CD31 immunostaining (Fig. 2, d and f). If all cases were included, no significant correlation was found between EPAS-1/HIF-2α expression and MVD (P = 0.422; Fig. 4A). Because superficial bladder cancers have a papillary structure with a fibrovascular core containing large vessels, we compared MVD with EPAS-1/HIF-2α expression in only invasive (pT2) cases. EPAS-1/HIF-2α-negative cases had significantly higher MVD than EPAS-1/HIF-2α-positive cases, and conversely, EPAS-1/HIF-2α-positive cases have lower MVD (P < 0.001; Fig. 4). To confirm this observation, we further analyzed serial sections from 23 EPAS-1/HIF-2α-positive cases with CD31 staining. MVD was assessed in 65 EPAS-1/HIF-2α-positive foci identified as the exact location for both EPAS-1/HIF-2α and CD31 staining. We also assessed MVD in 50 randomly selected EPAS-1/HIF-2α-negative foci, which contained stroma areas adjacent to tumor or necrosis in the same location. The spatial relationship between MVD and EPAS-1/HIF-2α in the perinecrotic/tumorous areas was assessed. A statistically significant inverse correlation (P < 0.001) was found, where EPAS-1/HIF-2α-negative foci had higher MVD than EPAS-1/HIF-2α-positive foci (Fig. 4C). Fig. 2, c–f showed examples of IHC staining in two sets of serial slides, demonstrating the typical inverse correlation of EPAS-1/HIF-2α with MVD in invasive bladder cancer.

**EPAS-1/HIF-2α Expression Is Associated with Focal TAM Infiltration in Perinecrotic Area.** Talks et al. (13) showed that EPAS-1/HIF-2α expression was seen in TAMs. To identify the location of EPAS-1/HIF-2α expression in bladder cancer tissues, we performed IHC against CD68 (the most common used macrophage marker, although not strictly tissue specific) and EPAS-1/HIF-2α Mab with serial sections. EPAS-1/HIF-2α- and CD68-positive staining coexisted in most but not all foci (Fig. 5, a and b). In these foci, fibroblasts stained positive for EPAS-1/HIF-2α (Fig. 5, c and d). To assess the correlation between Mφ1 and EPAS-1/HIF-2α positivity, CD68 immunostaining was done in all cases. As shown in Fig. 6, more TAM infiltration was found in EPAS-1/HIF-2α-positive cases than in EPAS-1/HIF-2α-negative cases (P < 0.001).
The major objective of this study is to demonstrate EPAS-1/HIF-2α expression correlated with hypoxia and necrosis in invasive bladder cancer, because intuitively, tumor, tumor metastasis, and tumor death should be closely correlated with tumor-induced hypoxia and necrosis. Cells in hypoxic regions are thought to be more resistant to the effects of radiotherapy and many conventional chemotherapeutic agents than their normoxic counterparts (for review, see Refs. 4 and 5).

In a series of parallel studies, we correlated the EPAS-1/HIF-2α overexpression with various clinicopathological parameters. Firstly, higher EPAS-1/HIF-2α expression was noted in the stroma cells palisading areas of tumor and necrosis, especially in tumors with large sizes. The perinecrotic/tumorous EPAS-1/HIF-2α expression is confirmed by IHC using both Mabs and Pabs, which were raised separately against different epitopes of the EPAS-1/HIF-2α immunogen (13) and also demonstrated by ISH based on gene transcription analysis. Secondly, perinecrotic/tumorous expression of EPAS-1/HIF-2α was correlated strongly with higher histological grades and pathological T stages (Table 2) and also with higher incidence of necrosis (Table 2). A parallel IHC study with an angiogenesis marker VEGF demonstrated that in more advanced bladder cancers, overexpression of both VEGF and EPAS-1/HIF-2α was coincidently found, supporting the notion that EPAS-1 expression is correlated with cancer invasion and progression in advanced bladder cancer.

Results derived from our statistical analysis of MVD and M/H9272 also provided some insight into the possible location and function of EPAS-1/HIF-2α expression in oncogenesis. Firstly, statistical data of our MVD assessment (Fig. 4) demonstrate where up-regulation of EPAS-1/HIF-2α expression was mostly identified; MVD was significantly lower than an EPAS-1/HIF-2α-negative area. This is another indication of hypoxic environment triggering EPAS-1/HIF-2α expression and the perinecrotic expression of EPAS-1/HIF-2α being apparently associated with hypoxia. Secondly, M/H9272 was positively correlated with EPAS-1/HIF-2α expression and with tumor grade and stage (Fig. 6). Furthermore, the function of EPAS-1/HIF-2α in cancer progression and invasion is possibly through focal TAM infiltration, which is localized near necrotic tumor areas, because we demonstrated that up-regulation of EPAS-1/HIF-2α near tumor and perinecrotic areas is associated with higher levels of TAM infiltration and is detectable mostly in macrophages (and some fibroblast cells).

It is increasingly evident that tumor angiogenesis requires the presence and contribution of stroma cells, especially TAM
cells (13, 22–28). In a transgenic mouse study with a VEGF promoter, strong transgenic expression induced by implantation of solid tumor was found in the stroma, but not tumor, indicating the important role of stroma cells in tumor microenvironment and angiogenesis (22). Recent studies indicated that TAM may play a central role in tumor angiogenesis and tumor progression, as they are capable of producing a large repertoire of angiogenesis factors, VEGF, tumor necrosis factor-α, and proteolytic enzymes.

Results from our analysis of Mvi and MVD in areas of hypoxia/necrosis and EPAS-1/HIF-2α expression are consistent with a number of reports from clinical studies on breast (23–25, 27, 28), ovarian (29), and lung (30) cancers and in hemangioblastomas (26). These reports all demonstrated that TAM “hotspots” were remotely located from the vascular hotspots of tumors, suggesting that TAMs may preferentially migrate toward areas of relative hypoxia (25). This in turn may attract TAMs into tumor, which then contribute to the angiogenic process, giving rise to association between high levels of angiogenesis and extensive necrosis (25). TAM may be attracted to necrotic tumors by chemotactic factors, such as VEGF (28, 31–33).

VEGF gene expression is induced by hypoxia and is considered essential for the growth and metastasis of solid tumors. As a potent proangiogenic cytokine, VEGF is reported to be overexpressed in both malignant tumors (27), stroma cells (22, 27, 33), and TAMs (28, 32, 33). Overexpression of VEGF is up-regulated in poorly vascularized (hypoxia) areas of breast carcinomas (23, 25, 27, 28). VEGF-positive TAMs are restricted to areas of VEGF production (28, 33). Evidence is accumulating that VEGF may be activated in stroma cells, especially in macrophages, with the process being mediated by the VEGF receptor flt-1 (31). Similarly, we observed up-regulation of VEGF protein in tumor foci with an avascular/hypoxic microenvironment and VEGF expression positively correlated with EPAS-1/HIF-2α overexpression. We found that the highest VEGF expression was detectable mostly in tumor areas, and only weaker staining in necrotic and stroma areas (some TAM cells) was detectable (data not shown). We would have anticipated positive staining of VEGF in TAMs, had more sensitive methodology, such as the tyramide signal amplification, been used as was reported recently (23, 28). Although bladder VEGF mRNA expression was reported to be higher in superficial than in muscle invasive bladder cancer (34), recent reports (35, 36) indicated that levels of VEGF mRNA and protein in bladder cancer tissues were very different and may be differentially regulated. In this study, we observed overexpression of VEGF protein in higher stage and grade of bladder cancers, which was confirmed with both Mabs and Pabs.

The specific signaling pathways involved in EPAS-1/HIF-2α activation, and the mechanism of the ensuing VEGF activation, are still unknown. On the basis of our results, we may assume that these processes should occur in hypoxic or necrotic areas through focal TAM infiltration. EPAS-1/HIF-2α was reported to be phosphorylated by a critical mediator p42/p44 mitogen-activated protein kinase through a mitogen- and stress-activated protein kinase pathway (37).

Similar to other HIFs (c-fos and cyclic AMP-responsive element binding protein), EPAS-1/HIF-2α protein is assumed to be accumulated on exposure of cells to hypoxia, then translocated to the nucleus, thereby trans-activating targeting genes containing the sequence of HRE. Evidence is accumulating that EPAS-1/HIF-2α may activate a series of genes known to be essential for angiogenesis, i.e., VEGF (6, 8), its receptor Flk-1 (38), and the endothelial receptor Tie 2 (1). These observations all point to a central role of EPAS-1/HIF-2α in blood vessel formation. We may postulate that EPAS-1/HIF-2α is first activated through focal TAM infiltration in the necrotic area adjacent to tumor foci, then binds to the HRE of the promoter of VEGF, triggering its expression.

Finally, we may expect a different mechanism for the activation of EPAS-1/HIF-2α in TAM in oncogenesis, because Talks et al. (13) have demonstrated that the majority of EPAS-1/HIF-2α protein and mRNA, along with VEGF expression, are all located in the cytoplasm of TAM in clinical cancer samples. This is in contradiction to the nucleus location shown in embryo development and in vitro cell culture tissue studies as reported previously (1, 2, 6, 8, 11, 13, 37, 39, 40). Furthermore, our detection of EPAS-1/HIF-2α-positive fibroblasts may indicate that stroma cells other than TAMs may also play a role in tumor angiogenesis.

Hypoxia response pathways may serve as a target for antitumor therapy (for review, see Ref. 4). Defective HIF-1α signaling and HRE-directed hypoxia targeting experiments have shown association with poor tumor growth in vivo and reduced angiogenesis (17, 40, 41). We expect demonstration of the clinical association of EPAS-1/HIF2α with hypoxia and necrosis may lead to exploration of its potential as a diagnostic tool and possibly a target for gene therapy for invasive bladder cancer.

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REFERENCES

Hypoxia-induced, Perinecrotic Expression of Endothelial Per-ARNT–Sim Domain Protein-1/Hypoxia-inducible Factor-2α Correlates with Tumor Progression, Vascularization, and Focal Macrophage Infiltration in Bladder Cancer

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