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Expression of pigment epithelium-derived factor and tumor necrosis factor-α is correlated in bladder tumor and is related to tumor angiogenesis

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Abstract

Objective: Angiogenesis is a pivotal process on which solid tumor growth is substantially dependent. Pigment epithelium-derived factor (PEDF) is the most potent natural anti-angiogenic factor, which has seldom been studied in bladder tumor, and whose functioning pathway remains unclear. We have thus investigated PEDF expression in relation to tumor necrosis factor-α (TNF-α) and microvessel density (MVD) with immunohistochemistry.

Methods: Antibodies of PEDF and TNF-α were examined by Western blotting before immunohistochemistry. Sixty-four urothelial tumor sections and 23 normal controls were stained and expression of PEDF, TNF-α, and MVD were studied.

Results: Decreased PEDF expression and increased TNF-α expression was noticed in tumorous tissue compared with healthy urothelium. Lower PEDF expression was related to higher tumor grade but stage. Increased TNF-α expression was noticed in recurrent, larger tumors as well as in tumors with progression in grade and stage. Expression of PEDF and TNF-α was correlated in bladder tumor. PEDF or TNF-α was correlated with MVD negatively or positively, respectively, in cancerous tissue and tumorous grouping without correlation in papillary urothelial neoplasm of low malignant potential.

Conclusion: Expressional change of PEDF and TNF-α is in relation to angiogenesis of bladder tumor, especially in bladder cancer development.

Keywords: Angiogenesis; Bladder tumor; Tumor necrosis factor-α; Microvessel density; Pigment epithelium-derived factor

1. Introduction

Urothelial cancer of bladder is the fifth most commonly diagnosed malignancy [1], taking up 3.2% among all cancers worldwide [2]. Despite this, the mortality rate of bladder cancer ranks twelfth; most patients have superficial tumors at the diagnosis au debut, but the recurrence of such disease is up to 70%, amid which 10%–30% of the patients suffer from progression in grade and stage [3]. Traditional surgical removal of either the tumor transurethral resection of bladder tumor (TURBT) or the whole bladder (radical cystoprostatectomy) meets predicament in further lowering recurrence rate or maintaining quality of life. Alternative treatments including radiation, chemotherapy, and photodynamic therapies substantially bring limited hope for patients with late staged cancer and works as alleviative therapy to certain extent. Angiogenesis has been extensively studied in bladder tumor and is believed to be critical in tumor growth and progression [4]. Unbalanced expression of pro- and anti-angiogenic factors in tumor condition results in neovas-

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cularization, which in turn promotes tumor growth, further loss of differentiation, and invasiveness.

Pigment epithelium-derived factor (PEDF) is one of the most potent natural anti-angiogenic factors premierly found in retinal tissue, maintaining avascularity. Aside from its neurotrophic and neuroprotective characteristics, PEDF has been reported to inhibit tumor growth in breast cancer [5], hepatocellular cancer [6], prostate cancer [7], and cervical cancer [8], either by antagonizing tumor angiogenesis or exerting direct inhibition. Nonetheless, PEDF has only been studied in bladder tumor by our institute so far [9]. In the previous investigation, we identified decreased PEDF expression in bladder tumors and negative correlation between PEDF expression and 2 pivotal proangiogenic factors, vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9).

In order to further understand the relationship between PEDF and angiogenesis in bladder tumor, we have involved in the present study the microvessel density (MVD) detection as well as another controversial yet crucial proangiogenic factor, tumor necrosis factor-α (TNF-α), which can, contrary to its name, be secreted endogenously by tumor cells causing chronic inflammation-related angiogenesis and can eventually promote tumor growth [10].The expression of both factors is quantified and their relations to angiogenesis are studied.

2. Materials and methods

2.1. Sample collection

Tumors from 44 transurethral resections and 17 cystoprostatectomies performed between 2007 and 2009 at Huashan Hospital were included with approval of local ethics committee. Patients' mean age was of 65.36 $\pm$ 13.87, which ranged from 21 to 87 years. A total of 23 healthy urothelium specimens were sliced from distant sites (>2 cm radius) in 23 cystoprostatectomy cases. Sections were restained with hematoxylin-eosin, graded and staged with WHO/ISUP (2004) [11] and UICC-TNM (2002) [12] by 2 independent pathologists. In this study, papillary urothelial neoplasm of low malignant potential (PUNLMP) was regarded as papillary lesions and was thus excluded from pT staging. Cancerous tissue was defined hereof as cases diagnosed with low grade or high grade bladder cancer and tumorous tissue was defined as PUNLMP plus cancerous cases.

2.2. Western blotting

Purified total protein was extracted from fresh urothelium and was applied at equal amount of 40 μg on sodium dodecyl sulphate polyacrylamide gel for electrophoresis. Proteins were then transferred to nitrocellulose membranes, which were subsequently blockaded with 1% bovine serum albumin for 4 hours. Mouse monoclonal antibody of PEDF (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit monoclonal antibody of TNF-α (Thermo Scientific, San Jose, CA) were then added at1:1000 and were cultured overnight. Goat anti-rabbit and rat anti-mouse polyclonal antibodies (Santa Cruz) were applied followed by application of horseradish peroxidase. β-actin was detected with goat monoclonal antibody (Santa Cruz). ECL Plus Kit (Amersham International, Buckingham, UK) was used for specific band detection.

2.3. Immunohistochemistry

Staining with PEDF antibody was processed in the method described previously [9]. TNF-α antibody was applied at 1:400 for boiled tissue sections in 10 mM citrate buffer, pH6, followed by similar procedures as PEDF. Microvessel was detected with application of rabbit polyclonal vWF (von Willebrand Factor) antibody (DAKO, Glostrup, Denmark) at 1:400 to a large glycoprotein confined within endothelial cells, megakaryocytes, and platelets, commonly used for microvessel detection. All slices were subsequently processed with DAKO Cytomation EnVision Doublestain System Kit (DAKO Corp., Carpinteria, CA) and with diaminobenzidine tetrahydrochloride for color development. Sections were eventually counterstained with Mayer's hematoxylin. For positive controls of PEDF and TNF-α, tissues of retinal pigment epithelial and skin were selected. For negative controls, slices applied with PBS (phosphate buffer solution) in lieu of primary antibodies were chosen.

2.4. Assessment

A Nikon 80i microscope was used for observation. Staining of PEDF and TNF-α was semiquantitatively evaluated by Ioachim et al. [13], namely extensity for PEDF 0 for 0%–5% of tumor cells stained, 1 for 6%–20%, 2 for 21%–50%, and 3 for >50% of cells stained, whereas for TNF-α, 0 for 0–10% cells stained, 1 for 11%–25%, 2 for 26%–50%, and 3 for >50% of cells stained. Intensity was unified as 1 for light yellow, 2 for dark yellow, and 3 for brown. Final score of each section comprised sum of both indicators, which was 0 for negative (1~2), 1 for mild (3), 2 for moderate (4), and 3 for strong (5~6). Microvessel density (MVD) was calculated by identification and quantification of vWF positive lumen cross-sections in 5 random fields per tumor at the magnification of $\times$200, as reported by Weidner [14].

2.5. Statistical methods

All data were presented as mean $\pm$ standard deviation (SD). For comparisons between 2 groupings, the Mann-Whitney *U*-test was used. For comparisons between 3 or more groupings, the Kruskal-Wallis test was applied. Correlations were studied with the Spearman test. A P value $<$ 0.05 was accepted as statistically significant.

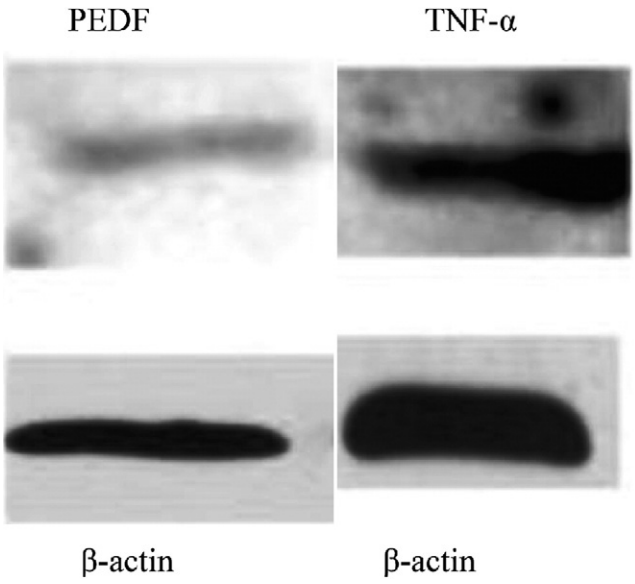

Fig. 1. Western blotting performed on fresh urothelium protein for specificity detection of the antibodies of pigment epithelium-derived factor (PEDF) and tumor necrosis factor-α (TNF-α) with internal control of β-actin.

3. Results

3.1. Western blotting and immunohistochemical stain

Specificity of primary antibodies of PEDF and TNF-α was demonstrated by western blotting in terms of the single bands shown in Fig. 1. Staining confinement of PEDF and TNF-α was chiefly cytoplasmic and nucleic, respectively (Fig. 2C, D). Microvessels could be identified by immunopositive endotheliocytes, which were stained brown in contrast to neighboring urothelial cells (Fig. 2A, B).

3.2. Groupings by clinicopathologic parameters

There were 48 males (75%) and 16 females (25%) included in the study, among whom 45 cases (70%) were detected single occurrence whereas 19 cases (30%) were multiple. Tumors less than 3 cm in size were identified in 41 cases (64%) and the rest, 23 were above 3 cm (36%). Forty-eight cases were diagnosed primarily (75%) and 16 cases were recurrent (19%). Tumors were graded as PUNLMP in 12 cases (19%), low grade cancer in 25 cases (39%), and high grade cancer in 27 cases (42%). Staging was evaluated within cancers in which 9 cases (17%) were pTa, 18 cases (35%) were pT1, 13 cases (25%) were pT2, and 12 cases (23%) were pT3+pT4.

3.3. Expressional relations of PEDF and TNF-α

There was no gender or occurrence pattern preference in PEDF or TNF-α expression (Table 1). Weak yet significant increase in TNF-α expression was noticed in larger and recurrent tumors (Table 1) in which PEDF expression did

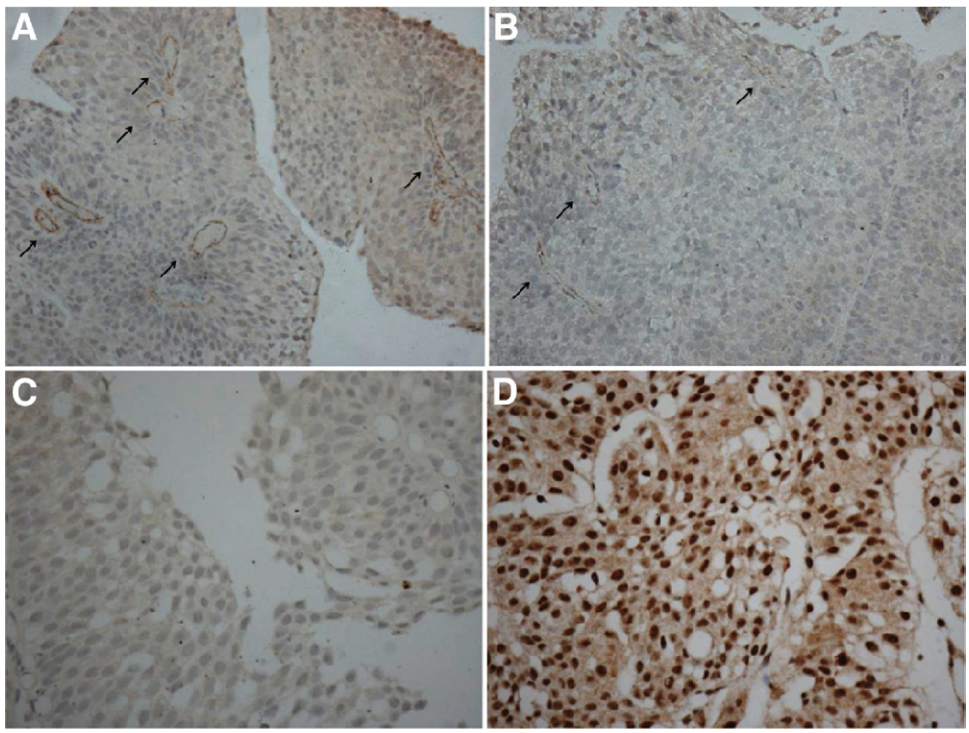

Fig. 2. VWF (von Willebrand Factor) positive endothelial cells indicating microvessels (arrows), denser in pigment epithelium-derived factor (PEDF) down-regulated section (A) and sparser in PEDF normally expressed section (B), captured at ×200. Cytosolic staining of PEDF in low grade bladder cancer (C) and nucleic staining of tumor necrosis factor-α (TNF-α) in high grade bladder cancer (D) are captured at ×400. (Color version of figure is available online.)

Table 1
Pigment epithelium-derived factor (PEDF) and tumor necrosis factor-α (TNF-α) expression in relation with clinicopathological parameters (mean ± standard deviation)

Parameters	*N*	PEDF	TNF-α
Sex			
M	48	0.9 ± 0.5	2.0 ± 0.9
F	16	0.8 ± 0.5	2.0 ± 0.9
P		0.804	0.862
Occurrence			
Single	45	0.9 ± 0.5	2.1 ± 0.8
Multiple	19	0.8 ± 0.6	1.8 ± 1.0
P		0.557	0.300
Tumor size			
<3 cm	41	0.9 ± 0.5	1.9 ± 0.9
≥3 cm	23	0.7 ± 0.5	2.3 ± 0.8
P		0.253	*0.018
Tumor onset			
Primary	48	0.9 ± 0.6	1.9 ± 0.9
Recurrent	16	0.8 ± 0.4	2.4 ± 0.7
P		0.879	*0.034
Tissue			
Normal	23	1.8 ± 0.6	1.1 ± 0.5
Tumorous	64	0.8 ± 0.5	2.0 ± 0.9
P		*0.000	*0.000
Normal	23	1.8 ± 0.6	1.1 ± 0.5
Papillary	12	1.2 ± 0.6	1.3 ± 0.8
P		*0.009	0.349
Grade			
PUNLMP	12	1.2 ± 0.6	1.3 ± 0.8
Low	25	0.9 ± 0.5	2.0 ± 0.9
High	27	0.6 ± 0.5	2.4 ± 0.8
P		*0.014	*0.000
Stage			
pTa	9	0.9 ± 0.3	1.7 ± 0.9
pT1	18	0.8 ± 0.6	1.9 ± 0.8
pT2	13	0.7 ± 0.5	2.6 ± 0.5
pT3+pT4	12	0.7 ± 0.5	2.6 ± 0.7
P		0.687	*0.005

* $P < 0.05$, statistically significant.

not significantly change. Decrease in PEDF expression and increase in TNF-α expression were identified in tumorous tissue compared with healthy urothelium (Table 1). Similar expressional change was also observed in PUNLMP concerning PEDF but TNF-α (Table 1). When tumors were grouped by grade, decreased PEDF expression and increased TNF-α expression were noticed (Table 1). Nonetheless, PEDF expression did not change significantly with stage progression whereas TNF-α expression elevated with more profound stage (Table 1). Cases were then grouped as papillary (PUNLMP, $n = 12$) and cancerous (low + high grade, $n = 52$), in which decreased expression of PEDF (1.2 ± 0.6 vs. 0.8 ± 0.5, $P = 0.029$) and increased TNF-α expression (1.3 ± 0.8 vs. 2.2 ± 0.8, $P = 0.008$) were found. When mere cancerous tissue was involved, expression of PEDF was decreased in high grade group ($n = 27$) (0.9 ± 0.5 vs. 0.6 ± 0.5, $P = 0.047$) and TNF-α expression was increased (2.0 ± 0.9 vs. 2.4 ± 0.8, $P = 0.040$) compared with low grade cancer ($n = 25$). Cases were then grouped as non-invasive (pTa, $n = 9$) and invasive (pT1+pT2+pT3+pT4, $n = 55$) in which expressional change of PEDF was not significant (0.9 ± 0.3 vs. 0.7 ± 0.5, $P = 0.392$) yet TNF-α expression slightly increased in the latter grouping (1.7 ± 0.9 vs. 2.3 ± 0.7, $P = 0.028$). When grouping of non-muscle-invasive bladder cancer (NMIBC) (pTa+pT1, $n = 27$) and muscle invasive bladder cancer (MIBC) was introduced, PEDF expression remained insignificant (0.9 ± 0.5 vs. 0.7 ± 0.5, $P = 0.260$), whereas expression of TNF-α was still elevated in MIBC (1.9 ± 0.8 vs. 2.6 ± 0.6, $P = 0.000$).

3.4. Correlation of PEDF, TNF-α, age and MVD

Neither PEDF ($P = 0.558$, $r = 0.07$) nor TNF-α ($P = 0.336$, $r = -0.123$) expression was correlated with age. The expressional correlation of PEDF and TNF-α was summarized in Table 2, in which a negative correlation was discerned when cases were grouped by tumorous as a whole. No correlation concerning PEDF and TNF-α was obtained when grouping was more specific as cancerous or papillary (Table 2). PEDF was revealed to be negatively correlated with MVD in both cancerous and tumorous groupings while TNF-α expression was positively correlated with MVD in such groupings (Table 2). Nonetheless, there was no correlation obtained in PUNLMP grouping concerning PEDF or TNF-α with MVD, as shown in Table 2.

4. Discussion

Angiogenesis has been emphasized for decades in search of the novel interventions against bladder tumor. It has been reported that solid tumor cease to grow in dearth of angiogenesis when tumor size reaches 2 mm in diameter [15].

Table 2
Correlation between expression of pigment epithelium-derived factor (PEDF), tumor necrosis factor-α (TNF-α), and microvessel density (MVD) in PUNLMP (papillary), bladder cancer, and altogether as bladder tumor

Objects	Papillary	Cancerous	**Tumorous
PEDF and TNF-α	$P = 0.748$, r = −0.09	$P = 0.126$, r = −0.22	*$P = 0.024$, r = −0.28
PEDF & MVD	$P = 0.128$, r = −0.46	*$P = 0.000$, r = −0.56	*$P = 0.000$, r = −0.56
TNF-α & MVD	$P = 0.846$, r = 0.01	*$P = 0.008$, r = 0.36	*$P = 0.000$, r = 0.53

* $P < 0.05$, statistically significant.
** Defined as papillary plus cancerous.

Pluripotent endogenous factor PEDF is a 50-kDa glycoprotein, which belongs to serpin family, exerting 4 major functions: neuro-protective and -trophic effect, antiangiogenic effect that inhibits tumor growth indirectly, direct inhibitive effect on tumor growth, and newly reported, anti-inflammatory effect [16]. As the most potent natural angiogenesis inhibitor, PEDF is highly selective in inhibiting remodeling vessels by inducing endotheliocyte apoptosis [17]. One of the commonly acknowledged pathways in which PEDF specifically blockades the angiogenesis is by antagonizing VEGF, and, another invasiveness related factor, MMP-9 [18]. Our institute has been the first in studying PEDF in bladder tumor and has revealed a negative correlation of PEDF, VEGF, and MMP-9 therein [9]. In the present study, we have further studied the relation between PEDF and microvessel density and have demonstrated a negative correlation in PEDF expression and angiogenesis of bladder cancers. The result is concordant with that in researches involving glioma [19], neuroblastoma [20], and prostate cancer [7].

Nonetheless, it is still unclear how distinctive factors interact in the antiangiogenic pathway PEDF mediates [18], and we have thus involved another potent proangiogenic factor, TNF-α, aiming for identifying whether the element functions in middle of the complicated pathway. TNF-α is a key cytokine modulating immunity, cellular organization, and inflammation. The conflicting roles of TNF-α as both a tumor necrosis and growth promoter have triggered a series of attempts of linking inflammation with carcinogenesis [10]. Current opinions on the dual function of TNF-α can be concluded as TNF-α induces hemorrhagic tumor necrosis depending on a pharmacologic high dose, which is accompanied by a series of adverse events. The TNF-α mediated cytotoxicity involves both innate and adaptive immune response [21] which, of note, requires an intact immune system where a series of down-stream cytokines like IL-2 or INF-γ can be activated [22], and T cells can subsequently eliminate the residual tumor cells [23]. Endogenous TNF-α, on the other hand, is much lower dosed and is a major component produced by many tumor cells. The secretion of endogenous TNF-α usually leads to chronic inflammation. Amid various effects TNF-α exerts in the tumor-promoting situation, its proangiogenic effect in favor of further stimulation of release of VEGF [24] etc. and stimulating effect of tumor remodeling via MMPs [25] have drawn much of our attention to the possible connection between TNF-α and PEDF.

There is a dearth of investigation concerning endogenous TNF-α expression in relation to PEDF or in bladder tumor. Zhang et al. [26] have reported down-regulation of PEDF is related to up-regulation of TNF-α in cultured retinal Müller cells and have indicated that PEDF also acts as an endogenous anti-inflammatory factor. In our study, expression of both factors was quantified by immunohistochemistry scoring. The results show the nucleic immunopositive confinement of TNF-α. Expression of TNF-α is significantly elevated in tumorous tissue compared with normal urothelium, tumors above 3 mm in size and recurrent tumors; also, higher expression is related to grade stage progression. Although the results imply that endogenous TNF-α may also exert tumor-promoting effect in bladder tumor, the selection of normal urothelium control may not be optimal in the current study. Although we have confirmed each 'malignancy-free' section as examined by 2 pathologists, there is still a chance that tumor milieu affects neighboring tissue to a certain extent. Samples from organ donors out with prior bladder disorders are better options despite the scarcity of sources. Further evaluations have revealed a positive correlation between TNF-α and MVD in tumorous tissue and cancerous tissue, indicating the possible tumor-promoting effect realized by angiogenesis. However, we fail to obtain any correlation between PEDF and TNF-α expression in either PUNLMP or bladder cancer, whereas a weak negative correlation thereof is attained when cases are grouped as tumorous totally. We assume that the angiogenic effect of TNF-α is exerted in a pathway which PEDF participates partially.

In the study of PUNLMP, merely a slight yet significant decrease of PEDF expression is noticed in comparison with healthy euroepithelium. Expression of TNF-α does not change in papillary lesions, nor does it correlate with PEDF or MVD in PUNLMP. According to the European Association of Urology (EAU), PUNLMP are papillary lesions that lack cytologic features of malignancy, yet the disease still has a tendency to recur [27]. The lack of malignant cytologic morphology in PUNLMP may be explainable in unchanged secretion of the tumor related factors and microvasculature, which is subsequently promoted by such factors.

5. Conclusion

Angiogenesis related factors PEDF and TNF-α have been proven to be pivotal in tumor growth. We have hereby discovered that decreased PEDF or increased TNF-α expression is related to differentiation, invasiveness, and angiogenesis of bladder tumor. The results should be informative for future development of targeted antiangiogenic therapy against bladder tumor.

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