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Distribution of EphB4 and EphrinB2 in normal and malignant urogenital tissue

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Abstract

Objective: Ephrin (Eph) receptors are receptor tyrosine kinases; both EphrinB2, as a ligand, and EphB4, as a receptor, are involved in angiogenesis. EphrinB2 is expressed on arteries and EphB4, a specific receptor for EphrinB2, is expressed on veins. It is unknown whether involvement of arteries and veins in tumor angiogenesis is distinctive. Here we investigated their distribution in normal and malignant tissue of the urogenital tract.

Materials and methods: Five-micrometer-thick paraffin sections from nontumoral and tumoral tissues of kidney ($n = 12$), bladder ($n = 33$), and prostate ($n = 20$) were immunoreacted with antisera against EphB4 and EphrinB2 using the avidin-biotin-peroxidase complex technique. Comparisons of EphB4 and EphrinB2 stained arterial and venous vessels in the nontumoral and tumoral sections were evaluated in a semiquantitative analysis as frequency of the vessels in a predetermined tumor area counted under light microscopy.

Results: Expression of EphrinB2 in arterial and EphB4 in venous endothelium was significantly greater in tumoral sections compared with nontumoral sections. No statistically significant correlation in comparing the labeling patterns for EphrinB2 with the labeling patterns for EphB4 was observed in nontumoral as well as tumoral sections.

Conclusions: The high expression of EphrinB2 in arterial and EphB4 in venous endothelium of urogenital tract tumors might contribute to their involvement in the progression of tumor angiogenesis. The relation between arteries and veins in the normal and tumor tissues is unchanged.

Keywords: EphrinB2; EphB4; Arterial angiogenesis; Venous angiogenesis; Tumor angiogenesis

1. Introduction

Blood vessels form via two distinct processes, vasculogenesis and angiogenesis [1,2]. Vasculogenesis involves in the differentiation of endothelial cells (ECs) from mesodermal angioblasts, which differentiate into endothelial cords [3]. In the angiogenesis, new blood vessels arise from the preexisting vasculature by proliferation and migration of endothelial cells. In the adult, angiogenesis is accompanied in the ovulation, placental development, and wound healing, and in the pathological processes such as tumor growth and metastasis, diabetic retinopathy, and rheumatoid arthritis [4,5].

The Eph receptors, which are a class of endothelial receptor tyrosine kinases, were implicated in the control of blood vessel formation [2,6,7]. An assembly of endothelial cells into capillary-like structures by promotion of EphrinB2 indicates that EphrinB2 may be involved in the angiogenesis [8]. These findings are supported by in vivo evidence that several disruptive embryogenic vasculature in the Ephrins-B2-deficient mice were observed [9]. In addition, the endothelial cell expression of EphB4 receptor suggested that EphB4 may mediate EphrinB2 signaling in the vascular development [9]. These evidences indicate clearly that Eph receptors and their Ephrin ligands are involved in the control of developing vasculature.

It has been reported that Eph/Ephrin are involved in the regulation of the tumor angiogenesis [10–12]. The distinct and specific involvement of arteries and veins in tumor

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angiogenesis is unclear. EphrinB2 is expressed in arteries and its receptor EphB4 is expressed in veins; these molecules play a prominent role in the control of angiogenesis. It was the purpose of our study to evaluate if tumor angiogenesis is associated with a distinct formation of arteries and veins.

Here we used tumor specimens of kidney, bladder, and prostate cancer to investigate the immunohistochemical expression of EphrinB2 in arteries and EphB4 in veins of tumor vessels. To study the endothelial localization of EphB4 and EphrinB2 in endothelium, double labeling of EphB4 and EphrinB2 with the endothelial marker PECAM-1 by immunofluorescence was carried out. EphB4 receptor was expressed in the tumor veins, whereas its ligand EphrinB2 was found in arteries. EphB4 expression and EphrinB2 expression of venous and arteries, respectively, were semiquantitatively compared in nontumoral and cancer tissue. Although there was a significantly higher expression of both markers in cancer tissue, no changes in the relationship of both markers were observed.

2. Materials and methods

2.1. Tissue obtaining

Tissue biopsy specimen of renal cell, transitional cell, and prostate cancer, and the adjacent normal tissue were obtained by open radical surgery in renal cancer ($n = 12$), prostate cancer ($n = 20$), and bladder cancer ($n = 33$). The stage of the patients with bladder cancer were pT2b (deep muscle infiltrated) and the grade was 2–3 (moderately to poorly differentiated) without metastasis and lymph node infiltration. The stage of the patients with prostate cancer were pT2b to pT2c (tumor involves more than a half of one lobe to involves both lobes) and the Gleason score 6 without metastasis and lymph node infiltration. The stage of the patients with renal cancer were pT1b (tumor $> 4 \leq 7$ cm in greatest dimension) without metastasis and lymph node infiltration.

2.2. Tissue preparation

Specimens were fixed in 4% paraformaldehyde for 4 hours and rinsed in 0.1 M PBS for 24 hours. The tissues were rinsed with 0.1 M PBS pH 7.4 buffer and stored in the same buffer at 4°C.

2.3. Paraffin embedding and section

The tissues were dehydrated in a graded series of ethanol dilutions (50%, 70%, 90%, 100%), then two changes of 100% chloroform, and embedded in paraffin. The paraffin sections were sectioned with a microtom at 5 μm, mounted on poly (L-lysine)-coated (0.1%) slides, deparaffinized through xylene, and rehydrated in descending concentrations of ethanol.

2.4. Frozen embedding and section

The tissues were cryoprotected with 20% sucrose solution, embedded in tissue-embedding medium, frozen in liquid nitrogen, and stored at −80°C until sectioning. Then, frozen embedded tissues were cut at 7 μm on a cryostat and mounted on poly (L-lysine)-coated (0.1%) slides.

2.5. Immunohistochemistry

The paraffin and frozen sections were incubated in methanol containing 3% H_2O_2 for 20 minutes at room temperature to inhibit endogenous peroxidase activity. Then the sections were incubated with 10% normal goat serum (NGS) + 1% bovine serum albumin (BSA) in 0.05 M TBS for 30 minutes at room temperature. The paraffin sections were incubated for 24 hours with primary antibodies anti-EphB4 (Santa Cruz, CA) and anti-EphrinB2 (Santa Cruz) at 4°C. The sections were incubated for 1 hour with biotinylated goat anti-rabbit IgG (DAKO, Hamburg, Germany) and for 1 hour with horseradish-peroxidase-complex (Amersham Biosciences, Piscataway, NJ). For visualization of the peroxidase reaction, the sections were incubated in a solution containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma, Taufkirchen, Germany), 0.01% nickel ammonium sulphate and 0.05% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6, for 10 minutes at room temperature. The sections were dehydrated in increasing concentrations of ethanol, cleared in xylene, and mounted with Entellan (Merck AG, Darmstadt, Germany).

2.6. Double immunofluorescence

The sections were incubated at RT with a cocktail mix of 2 primary antibodies: mouse monoclonal anti-PECAM-1 (1:400) with rabbit polyclonal anti-EphB4 or mouse monoclonal anti-PECAM-1 (1:400) with rabbit polyclonal EphrinB2. Subsequent antibody detection was carried out with a cocktail mix of 2 secondary antibodies, CY2-conjugated anti-mouse IgG and CY3-conjugated anti rabbit-IgG. The sections were washed in 0.05 M TBS and mounted with Entellan.

2.7. Immunohistochemical controls

Incubations without primary or secondary antibodies were carried out as immunohistochemical controls.

2.8. Semiquantitative analysis

EphB4 and EphrinB2 stained arterial and venous vessels in the normal and tumorous tissue were evaluated and compared in a semiquantitative fashion. In order to define the number of