ORIGINAL PAPER

Immunohistochemical expression of VEGF in normal human renal parenchyma

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Abstract

In the normal kidney, VEGF is constitutively expressed in podocytes and tubular epithelial cells of the renal cortex and medulla. The aim of this study was to determine distribution of VEGF in normal renal parenchyma using immunohistochemical methods. The study was retrospective, using normal kidneys samples taken from 28 patients with nephroureterectomy for different types of renal cell carcinomas. Sections were stained with routine Hematoxylin–Eosin method and immunohistochemically with anti-VEGF polyclonal antibody. All cases presented an intense immunoreaction in the cells lining the nephron tubular system, the higher immunoreaction intensity in the collecting and distal tubules, but weak or moderate in the proximal tubules. The Henle loop cells showed a negative immunoreaction. The immunoreaction was absent in most cells of the renal corpuscle. The cells lining the same tubule presented some variation of intensity, with large polygonal epithelial cells, bulging in the tubular lumen showing an intense cytoplasmic immunoreaction for VEGF. In the renal parenchyma adjacent to the tumor, we observed the same pattern of positive reaction distribution as in the nephron's epithelial tubular cells situated far from the tumor. Adjacent to the tumor proliferation front and in those cases with massive invasive features, we observed a partial depletion of VEGF in distal tubules, while the majority of collecting ducts remained intense positive. The VEGF immunostaining was significantly higher in the renal cortex than in the outer and respectively the inner medulla.

***Keywords*:** vascular endothelial growth factor A, kidney, distal tubules, collecting ducts, podocytes, immunohistochemistry.

Introduction

Vascular permeability factor (VPF) or vascular endothelial growth factor A (VEGF–A) is a member of a family of dimeric glycoproteins that belong to the platelet-derived growth factor (PDGF) superfamily of growth factors. Other members of the VEGF family include VEGF–B, VEGF–C, VEGF–D, VEGF–E and placenta growth factor, PlGF [1–3].

Discovered in the late 1970s as a tumor-secreted protein that increased microvascular permeability to plasma proteins, VEGF was defined as a homodimeric heparin-binding protein, with a molecular weight of 45 kDa. It was first isolated and purified from media conditioned by bovine pituitary follicular stellate cells [4].

In 1983, Senger DR *et al.* [5] described the partial purification of a protein able to induce vascular leakage in the skin, which was named "tumor vascular permeability factor" (VPF). The authors proposed that VPF could be a mediator of the high tumor blood vessels permeability. Because VPF was not isolated and sequenced, this factor remained molecularly unknown at that time. Senger DR *et al.* [6] reported the purification and amino-terminal amino acid sequencing of guinea pig VPF in 1990.

In 1989, Ferrara N and Henzel WJ [4] reported the isolation of a diffusible endothelial cell-specific mitogen from medium conditioned by bovine pituitary follicular cells, which they named "vascular endothelial growth factor" to reflect the restricted target cell specificity of this molecule. This protein was distinct from the known endothelial cell mitogens such as aFGF or bFGF [7].

Subsequently, in 1989, Connolly DT *et al.* [8] followed up on the work by Senger DR *et al.* [6] and independently reported the isolation and sequencing of human VPF from U937 cells. cDNA cloning of VEGF and VPF, demonstrated that VEGF and VPF were the same molecule.

The finding that VEGF is potent, diffusible, and specific for vascular endothelial cells led to the hypothesis that this molecule might play a role in the regulation of physiological and pathological growth of blood vessels [9, 10].

Vascular endothelial growth factor A has six isoforms that bind to high affinity receptors, predominantly located on vascular endothelium, through which it induces endothelial cell proliferation and increases vascular permeability to different macromolecules.

The major isoform is the VEGF165, a basic, heparin-binding, homodimeric glycoprotein [11]. VEGF121 is a weakly acidic polypeptide that fails to bind to heparin. VEGF189 and VEGF206 are more basic and bind to heparin with greater affinity than VEGF165. Previous studies demonstrated that such differences in the isoelectric point and in affinity for

heparin may profoundly affect the bioavailability of VEGF.

VEGF121 is a freely soluble protein, but a significant fraction of VEGF165 remains bound to the cell surface and the extracellular matrix. In contrast, VEGF189 and VEGF206 are almost completely sequestered in the extracellular matrix. However, these isoforms may be released in a soluble form by heparin or heparinase, suggesting that their binding site is represented by proteoglycans containing heparin-like moieties [12].

In contrast to its transient-expression during the formation of new blood vessels, VEGF and its receptors are continuously and highly expressed in adult tissues, such as the kidney and choroid plexus.

VPF/VEGF contributes to angiogenesis by both direct and indirect mechanisms. On the one hand, VEGF stimulates the endothelial cells to proliferate, to migrate, and to alter their pattern of gene expression. On the other hand, VEGF induces microvascular hyperpermeability generating a provisional plasma-derived matrix for the ingrowths of new blood vessels.

The aim of this study was to determine distribution of VEGF in normal renal parenchyma using immunohistochemical methods.

Material and methods

Specimens

Normal human kidney samples were obtained from 28 patients with different type of renal cell carcinomas, admitted in the Clinical Hospital of Timişoara. The surgical technique performed in all patients was nephroureterectomy, so that in each case specimens were taken from the tumor, kidney and ureter. Specimens were fixed in 4% buffered formalin, embedded in paraffin. Sections were stained with routine Hematoxylin–Eosin method.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue derived from the normal pole of the nephrectomy specimens, using the three steps labeled streptavidin–biotin–immunoperoxidase technique (LSAB2, DAKO, Glostrup, Denmark).

Five-micrometer thick sections were cut and mounted onto poly-L-lysine–coated glass slides. Sections were dewaxed, rehydrated, washed in distilled water, and then rinsed in 0.01 mol/L Tris-buffered saline (PBS, pH 7.2).

The antigen retrieval consisted of microwave heating in DakoCytomation Target Retrieval Solution, pH 9 at 800 watts for two cycles of 10 minutes, followed by double washing with distilled water for 5 minutes.

After endogenous peroxidase inhibition with 3% hydrogen peroxide solution, the sections were washed twice with PBS and then incubated with the first step antibody: polyclonal mouse anti-human vascular endothelial growth factor antibody, clone VG1, in a 1 : 50 dilution. This antibody labeled all three isoforms of VEGF: VEGF–121, VEGF–165 and VEGF–189.

The slides washed twice in PBS, consecutively reacted with a labeled streptavidin–biotin system (DAKO LSAB+/HRP kit). The reaction product was visualized in brown with diaminobenzidine (DAB) as a chromogen.

Sections were washed twice in distilled water for 5 minutes, which stopped the reaction, then counterstained in Hematoxylin for 5 minutes, dehydrated, cleared in xylene, mounted with DPX, and glass cover slipped.

Sections were examined under oil immersion with a ×40 objective on a Nikon Eclipse E–400 microscope, and images were captured using a Coolpix 995 digital camera and a DN–100 digital imaging system (Nikon).

Anti-vimentin (clone V9) antibody was used as a marker of the optimal fixation and embedding procedures. The negative control was a nonspecific immunoglobulin, provided by the manufacturer (DAKO, Denmark), and performed on slides from the same cases and in the same concentration as the primary antibody. The immuno-labeling of tumor cells had been used as intern positive control. All reagents for the immunohistochemical technique were supplied from DAKO, Glostrup, Denmark.

Results

VEGF expression

The presence of VEGF was demonstrated immunohistochemically, using a polyclonal anti-VEGF antibody (clone VG1). There were studied only biopsies taken from both, tumor and renal parenchyma The reaction product was visualized in brown in both, normal or tumor immunopositive cells, with cytoplasmic localization, granular pattern and a higher concentration in the perinuclear area (Figure 1).

The expression of VEGF in normal renal parenchyma distant from tumor

All cases presented an intense immunoreaction in the cells lining the nephron tubular system. The intensity of the immunoreaction was higher in the collecting ducts and distal tubules, but weak or moderate in the proximal tubules.

The majority of Henle loop cells showed a negative immunoreaction, but some isolated cells of the Henle loop were positive, and presented a weak, focal immunoreaction.

The immunoreaction was negative in the glomerulus and in most cells of the renal corpuscle. In only one case, we observed a weak positive immunoreaction for VEGF in some isolated podocytes. Isolated positive cells, localized in the outer parietal epithelium of Bowman's capsule, have been also observed on a few slides (Figure 2).

Small clusters of epithelial cells, localized in the outer parietal layer of Bowman's capsule, at the urinary pole, showed an intense positive immunoreaction.

A similar staining pattern was observed in the epithelial cells of the proximal convoluted tubules (Figure 3).

The reaction intensity was higher in the distal tubules epithelial cells, situated at the vascular pole of glomerulus. The intensity of VEGF expression varied along the different segments of the tubular system. It was higher in collecting ducts and distal tubules epithelial cells than in those of nephron's proximal tubules (Figures 4 and 5).

Even if the intensity of reaction was almost uniform in the tubules, in transversal section, cells lining the same tubule presented some variation of intensity, due to the presence of some large polygonal epithelial cells, bulging in the tubular lumen that expressed an intense but diffuse cytoplasmic immunoreaction for VEGF (Figure 6).

Such an intense positive reaction, cited also by other authors, could be related to one of the most important functions of VEGF, the increasing of vascular permeability.

It is known that VEGF was initially named vascular permeability factor. The strong positive immunoreaction for VEGF in the collecting ducts and distal tubules epithelial cells suggest a relationship between VEGF expression and adjacent straight vessels (*vasa recta*), with important roles in gradient concentration. The high levels of VEGF can be correlated with the particular evolution of renal tumors, because the pre-existing VEGF in renal parenchyma represents a local source of growth factor.

VEGF expression in renal parenchyma adjacent to the tumor

Renal parenchyma showed a consistent VEGF staining in the nephron's tubular system, with no evidence of positive immunoreaction in the renal corpuscle or renal interstitium (Figure 7).

Regarding to the intensity of VEGF immunostaining, we observed, in the renal parenchyma adjacent to the tumor, the same pattern of positive reaction distribution as in the nephron's epithelial tubular cells situated far by the tumor. The positive reaction was more intense in the epithelial cells of distal tubules and collecting ducts than in those of the proximal tubules. The collecting ducts epithelial cells labeled for VEGF with some variation of positive reaction, while the Henle loop cells kept a negative or very weak positive reaction for VEGF (Figure 8).

The VEGF immunostaining was significantly higher in the renal cortex than in the outer and respectively the inner medulla.

In the renal parenchyma, adjacent to the tumor proliferation front, and in those cases with massive invasive features, we observed a partial depletion of VEGF in distal tubules epithelial cells, while the majority of collecting ducts were still intense positive (Figure 9), characteristics not mentioned by other authors.

The depletion of the reaction product in tubular epithelial cell cytoplasm was associated with changes in tubular architecture and with the presence of a partially tubules atrophy. These changes were constant in those cases associated with glomerular sclerosis, where the tubular cells of sclerotic glomeruli were negative for VEGF.

In one case of clear cell renal carcinoma, we observed the presence of isolated tubules surrounded by the tumor proliferation (Figure 10). The tubules were identified according to their apparent normal morphology. The positive immunoreaction for VEGF was significantly higher in the epithelial tubular cells within the tumor than in tumor cells (Figure 11). This pattern of VEGF expression was similar for normal tubules within tumor area and for small clusters of epithelial tubular cells, localized between tumor cells, staining negative or weak positive for VEGF (Figure 12).

Discussions

The human VEGF-A gene is located on chromosome 6 and contains eight exons separated by seven introns. There are six different isoforms named after the amino acids number of the peptide chain, VEGF–121, VEGF–145, VEGF–165, VEGF–183, VEGF–189 and VEGF–206 [12, 13].

VEGF–165 is the most abundantly expressed isoform [14] and VEGF–206 the rarest [13, 15].

VEGF mediates its biological effects by binding to more than two receptors belonging to the family of tyrosine kinases [10]. The VEGF A receptors initially described were VEGFR–1 (Flt–1) and VEGFR–2 (KDR, flk–1) localized on the microvascular endothelium of the normal kidneys and tumors, healing wounds and inflammatory sites [16–18]. The third receptor for VEGF A has been later demonstrated to be neuropilin–1 [13, 19]. The expression of neuropilin–1 by normal human podocytes was shown on studies in vitro and in vivo [17, 20, 21]. VEGF has been shown to reduce apoptosis in a large number of cells and cell lines, many of which express VEGF–R1. The complex, formed by the tumor secreted VEGF and its receptors, may become a potential target for antiangiogenic therapy [22, 23].

VEGF has been demonstrated to have an important role in kidney organogenesis, especially glomerulogenesis, acting as a paracrine messenger molecule between capillary endothelial cells and Bowman's capsule, in order to maintain the glomerular integrity [24].

In human embryonic and adult kidney, VEGF was localized in the glomerular epithelial cells, while its receptors were present in endothelial cells of both glomerular and peritubular capillaries [24].

Some authors studied the VEGF expression during embryonic development and discovered that is first

expressed in the trophoblast, within the first days after implantation, suggesting a role for this factor in the angiogenesis in the decidua and placenta. In the human fetus (16–22 weeks), VEGF mRNA expression was present in many tissues and was highly expressed in the lungs, kidneys, and spleen [12, 25].

Previous studies have shown that VEGF is continuously expressed in organs with fenestrated endothelia such as choroid plexus and kidney glomeruli [26].

In some studies has been emitted the hypothesis that the continuous expression of VEGF is some particular tissues might be involved in the induction or maintenance of endothelial fenestrations [7, 27, 28]. In most adult tissues, the VEGF positive reaction present during embryonic vasculogenesis and angiogenesis is down-regulated parallel with the reduction of endothelial proliferation.

Using immunohistochemical methods, the VEGF presence has been identified in some epithelial cells, kidney glomeruli and myocytes, but not in vascular endothelial cells [26]. On human kidney, a positive VEGF reaction product was demonstrated in visceral glomerular epithelial cells, also known as podocytes [29, 30]. Normal human podocytes are also known to express VEGF receptors such as neuropilin–1, but are VEGF–R2 negative.

The main VEGF isoform expressed by podocytes is VEGF165. Other VEGF isoforms such as 121, 165, and 189 has been also demonstrated in activated mesangial cells, during mesangioproliferative nephritis.

Other authors demonstrated that in kidney glomeruli the major site of VEGF expression was the podocyte [31] but its functional role and the factors responsible for its regulation are poorly understood and controversial [27, 32, 33]. Vascular endothelial growth factor seems to be anti-cytotoxic in podocytes. Moreover, it has been suggested that nephrin, a cell adhesion molecule of the podocyte slit diaphragm, can contribute to antiapoptotic mechanisms in these cells [21].

In our study, opposite with data shown by other authors, the majority of renal corpuscle cells showed no positive reaction for anti-VEGF antibody. The lack of positive immunoreaction in glomerular cells was similar for the renal corpuscle adjacent to the tumor or situated distant from tumor. We identified only a small number of visceral glomerular epithelia cells VEGF immunopositive, but the immunoreaction intensity was weak and restricted.

In comparaison, Dvorak HF *et al.* [34] identified a strong VEGF expression in glomerular podocytes and tubular epithelium of normal kidney [29, 34]. In 2003, Eremina V [35] demonstrated the VEGF expression in the podocytes of normal human kidney, the epithelial cells of the distal tubules and collecting ducts.

Using immunohistochemical and in situ hybridization methods, the VEGF mRNA and protein have been co-localized in glomerular epithelia and collecting duct cells.

Some studies revealed that mature podocytes express significant levels of distally spliced isoforms of VEGF that have been shown to be inhibitory and antiangiogenic [36]. The polyclonal antibody used in our study binds both isoforms of VEGF: 165 and 165b, but even so we did not find a positive reaction in glomerular cells.

Regarding the expression of VEGF in the cells of Bowman's capsule, we identified a negative expression in the majority of Bowman's capsule outer epithelial cells, with only few isolated positive parietal cells. The immunoreaction was moderate at urinary pole, present in small clusters of intense positive parietal epithelial cells, localized adjacent to the proximal tubule.

In conformity with the hypothesis emitted in 1997 by Kitamoto Y [24], Bowman's capsule secretes VEGF in order to stimulate capillary endothelial cells to develop a glomerulus.

In the nephron's tubules, the upregulation of VEGF is initiated also by the hypoxic signal resulting from poor peritubular vascularity. In distal tubules, VEGF expression may be necessary to maintain the fenestration of capillary endothelial cells.

Some authors have demonstrated other VEGF positive cells, such as: mesangial cells [37], glomerular endothelial cells [31], activated macrophages [37], renal glomerular visceral epithelia [29]. These observations suggested that VEGF might play an important regulatory role for glomerular endothelial cells [31, 38].

Opposite to these studies, we did not identify any VEGF positive mesangial cells by immunohistochemistry.

The distal tubules and collecting ducts showed a strong positive reaction, these findings being similar with data of Brown LF *et al.* [29] that identified the VEGF protein in the epithelia of collecting ducts. In 1997, Kitamoto Y [24] highlighted the evidence of VEGF message in proximal and distal convoluted tubules of the adult rat.

Previous immunohistochemical studies have provided solid evidence of VEGF presence in normal human kidney, confined to distal tubule and collecting ducts [18, 34].

The expression of VEGF in collecting ducts and of VEGF receptors in medullar capillaries may play a role in maintaining the medullar osmolarity. VEGF may promote renal tubular epithelial cell survival in vivo in situations associated with cellular stress, for example acute ischemia or toxic injury of the kidney. These data suggest an expanded role for VEGF in pathological conditions in the kidney [39].

Our study revealed that even if the positive reaction for VEGF had a constant presence in normal renal parenchyma, its intensity varied along the different segments of the tubular system. It was higher in epithelial cells of the collecting and distal tubules, with minimal reaction in proximal tubules of the nephron. The Henle loop epithelial cells showed no VEGF staining.

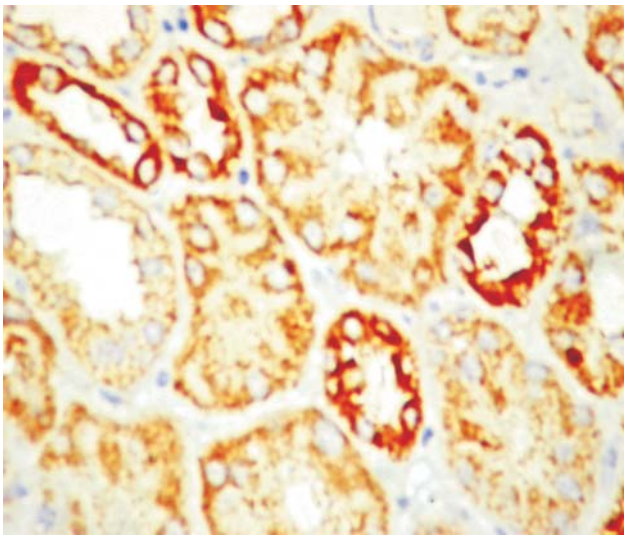

Figure 1 – *Cytoplasmic positive immunoreaction for VEGF with a higher perinuclear concentration and a granular pattern in normal renal tubules. Immunoreaction for VEGF (×400)*

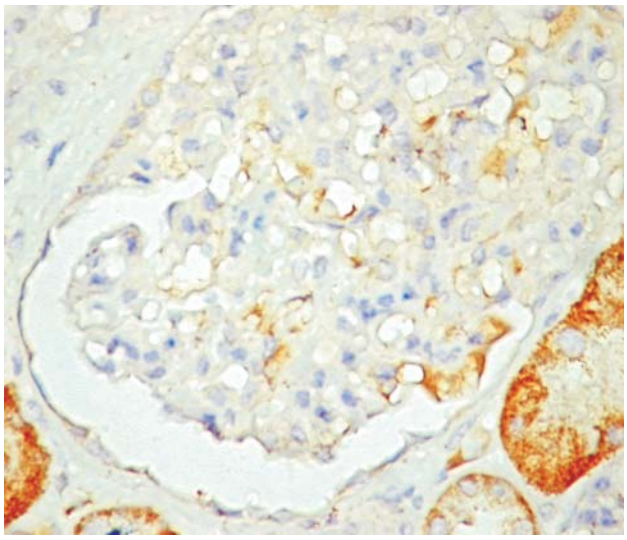

Figure 2 – *Immunoreaction for VEGF in the renal corpuscle. Weak positive reaction in some isolated or small clusters of podocytes (×400)*

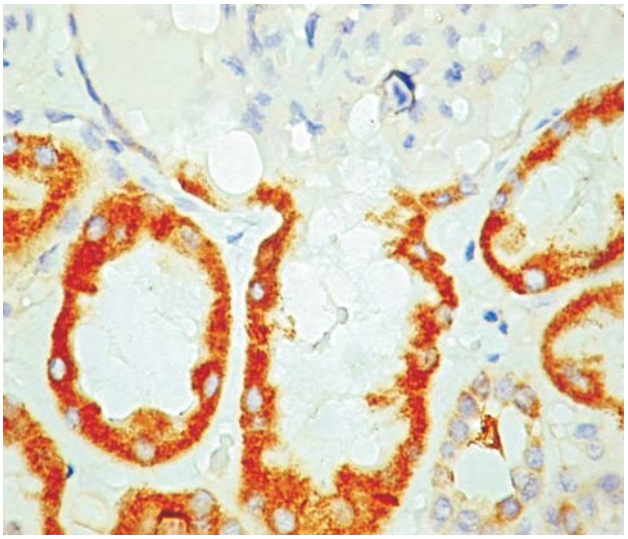

Figure 3 – *Urinary pole of renal corpuscle Proximal convoluted tubule epithelial cells showed an intense positive reaction. Immunoreaction for VEGF (×400)*

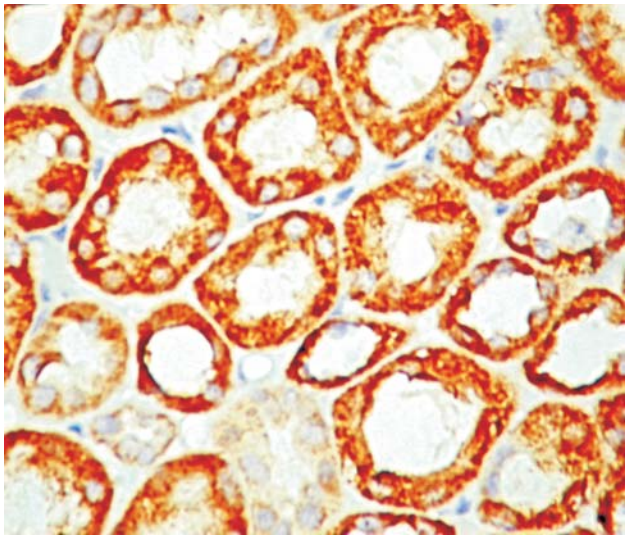

Figure 4 – *Intense immunostaining in distal and collecting tubules of the outer medulla. Immunoreaction for VEGF (×400)*

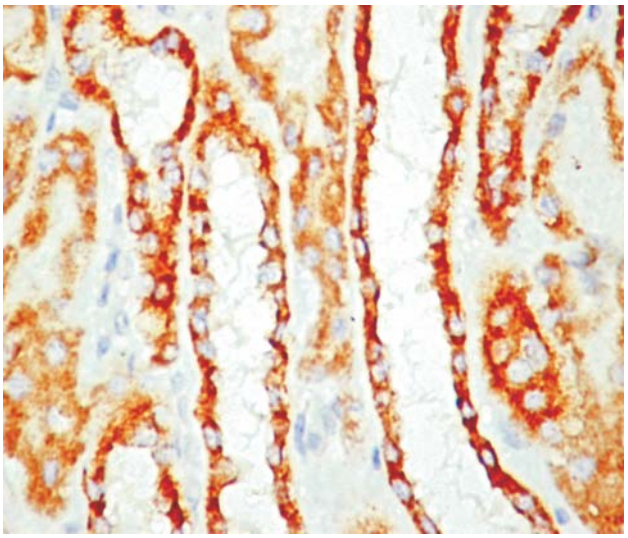

Figure 5 – *Proximal tubules of the outer medulla with weak positive reaction. Immunoreaction for VEGF (×400)*

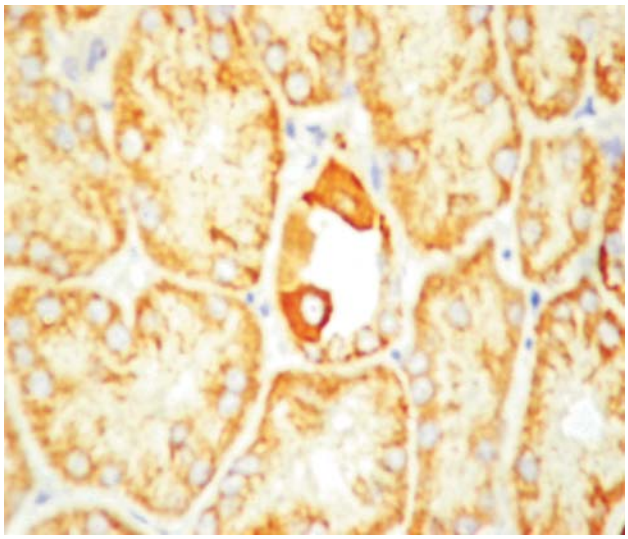

Figure 6 – *Heterogenic positive immunoreaction in tubular epithelial cells (×400)*

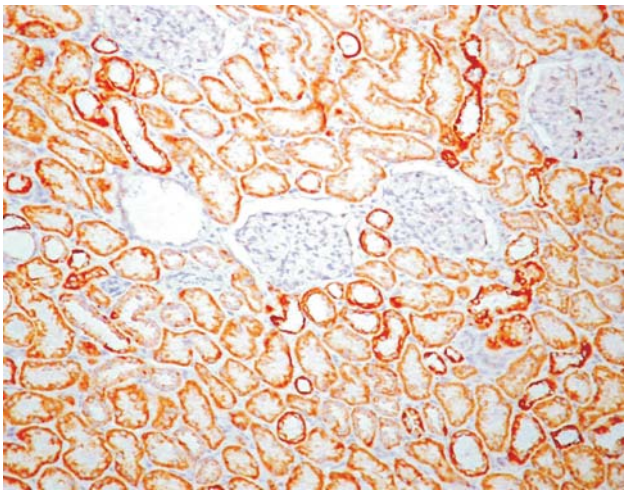

Figure 7 – ***Normal kidney with diffuse immunohistochemical staining for VEGF in all tubules of the renal cortex, and no staining of renal corpuscle or renal interstitium (×100)***

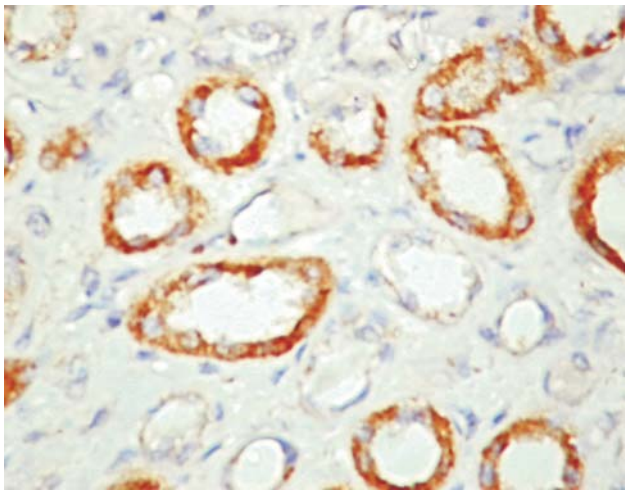

Figure 8 – ***Collecting ducts with intense positive cells, and Henle loop epithelial cells with negative immunoreaction for VEGF (×400)***

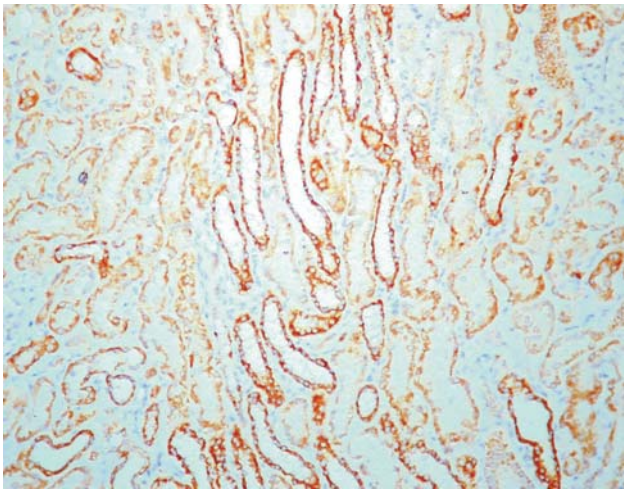

Figure 9 – ***The partial depletion of VEGF in distal tubules and the Henle loop. The majority of collecting ducts were still intense positive (×100)***

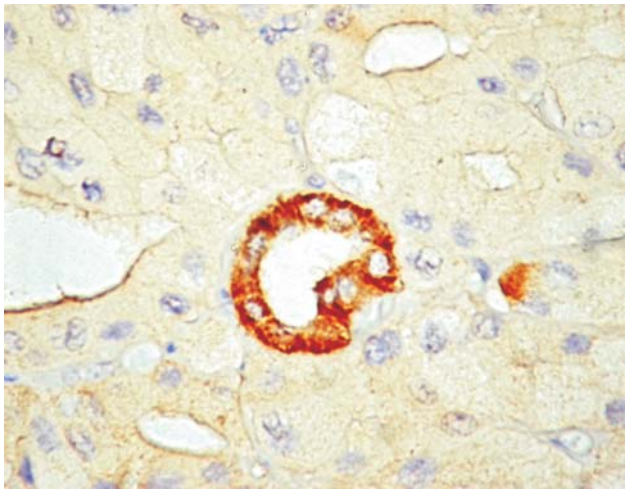

Figure 10 – ***Renal tubule with apparent normal morphology within the tumor. Immunoreaction for VEGF (×400)***

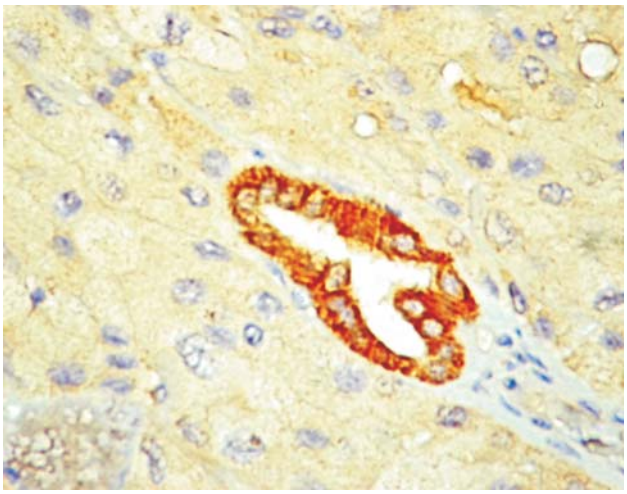

Figure 11 – ***The intensity of positive reaction is higher in tubular cells within the tumor than in tumor cells). Small cluster of tubular cells, intense positive for VEGF and tumor cells weak positive. Immunoreaction for VEGF (×400)***

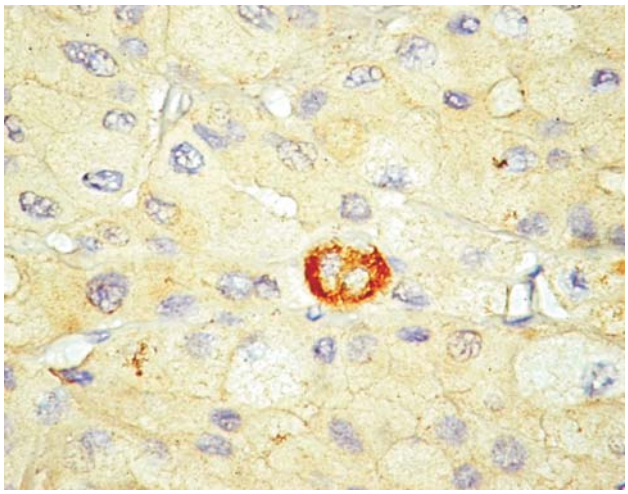

Figure 12 – ***Small cluster of tubular cells, intense positive for VEGF and tumor cells weak positive. Immunoreaction for VEGF (×400)***

There are no other studies reporting about the VEGF presence in the renal parenchyma adjacent to the tumor. In our study, we identified a partial depletion of VEGF in distal tubules epithelial cells, while the majority of collecting ducts remained intense positive. In addition, the presence of apparent normal tubules within the tumor area could reflect the high rate of tumor proliferation, but these aspects need to be further investigated.

One researcher group that have investigated the expression of VEGF in human renal tissues, have found an increased VEGF staining only in the tumor area and in surrounding vascular tissue, but not in the area of normal renal tissue. Although these studies included a relatively small number of patients, the decreased expression of VEGF, support the hypothesis that in patients with minimal change disease there was a specific down-regulation of VEGF gene expression [7].

We consider that the differences noticed between our results and those published by other researcher groups can be explained by the type of antibody (polyclonal versus monoclonal) and by the used method for the VEGF determination (immunohistochemistry versus polymerase chain reaction or in situ hybridization).

Conclusions

Our study confirms the presence of VEGF in the nephron's tubular system situated adjacent or distant from tumor.

The VEGF immunoreaction was intense in the epithelial cells of the distal tubules and collecting ducts and moderate in the proximal tubules epithelial cells. The Henle loop epithelial cells were VEGF negative.

The cells of renal corpuscle did not react with the polyclonal anti VEGF antibody.

Although recent studies have identified an intense positive reaction for VEGF in podocytes, our study revealed only a few podocytes with a weak positive reaction and a minimal positive reaction in some parietal epithelial cells of the Bowman's capsule. This positive reaction, in some podocytes and parietal epithelial cells, was observed in a scarce number of cases.

On one slide, along the tumor proliferation front, there was observed the presence of an apparent normal tubule with positive VEGF cells surrounded by weakly stained tumor cells.

The same positive immunoreaction was observed even in small clusters of normal tubular epithelial cells within the tumor.

In the peritumoral area, the proximal and distal tubules epithelial cells showed a less intense positive immunoreaction for VEGF than those localized in renal parenchyma situated distant from tumor.

The immunoreaction intensity for VEGF decreased from kidney cortex to inner medulla.

The glomerular sclerosis has been associated with a negative reaction in the epithelial tubular cells.

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