Expression of vascular endothelial growth factor and its receptors in rats with protein-overload nephrosis

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Abstract

Background. Based on the fact that vascular endothelial growth factor (VEGF) increases vascular permeability, it is speculated that VEGF might be involved in the development of proteinuria, although this remains unconfirmed. The production and site of action of VEGF remains unclear in nephrotic renal diseases.

Methods. Non-radioactive in situ hybridization was performed to examine the expression of VEGF mRNA and its receptors, flt-1 and KDR/flk-1, in a rat model of nephrosis induced by intraperitoneal injection of bovine serum albumin (BSA). Saline injected rats were served as control animals.

Results. Neither morphological changes nor deposition of immunoglobulin or complement were observed in our model. Proteinuria developed, reaching a maximum level in rats injected with BSA for 3 days, followed by persistent proteinuria until day 14. The expression of mRNA for VEGF and the two receptors was markedly upregulated in glomeruli of BSA-induced nephritis compared with the control group. VEGF mRNA was localized in glomerular cells, including cells in mesangium, visceral and parietal epithelial cells. In contrast, flt-1 mRNA and KDR/flk-1 mRNA were expressed on glomerular endothelial cells and cells in mesangium. The ratio of glomerular cells positive for VEGF mRNA and its receptors mRNA increased proportionately with the severity of proteinuria. Immunohistochemistry for ED-1 and proliferating cell nuclear antigen showed no significant increase in infiltrating macrophage or cellular proliferation.

Conclusions. Our results suggest that altered glomerular expression of VEGF and its receptors is not associated with proliferation of endothelial cells, but rather with proteinuria in BSA-induced nephritis in rats. VEGF may play a different role in different renal diseases.

Key words: VEGF; flt-1; KDR/flk-1; proteinuria; in situ hybridization; rats

Introduction

Vascular endothelial growth factor (VEGF)/vascular permeability factor has pleiotropic functions [1–5]. Based on its enhancing activity vascular permeability, VEGF is thought to be involved in the development of proteinuria. Identification of the site of VEGF production and receptor in renal tissue is important for a better understanding of the role of VEGF in renal diseases. However, the expression of VEGF and its receptors in nephrotic renal diseases are not fully understood. Using radioactive in situ hybridization, several studies showed that the expression of VEGF mRNA is limited to glomerular epithelial cells [3–5] and that such expression was reduced in sclerotic lesions of human renal diseases [7,8]. On the other hand, Iijima et al. [9,10] reported that cultured glomerular mesangial cells were capable of producing VEGF. With regard to the receptor, two receptors for VEGF, flt-1 and KDR/flk-1, have been recently identified [11–13]. In anti-Thy1 nephritis, a model of mesangial proliferative glomerulonephritis, only KDR/flk-1 mRNA is expressed [14], but the expression of VEGF and its two receptors in other renal diseases has not been investigated.

A variety of cytokines and growth factors are involved in the pathogenesis of different forms of glomerulonephritis [15,16]. Several cytokines and growth factors, such as TGF-β, PDGF, IL-6, IL-1β are involved in mesangial proliferation and TGF-β and PDGF increased VEGF [9,17,18] and flt-1 [17] expression. These findings suggest that in renal diseases with morphological change, such as mesangial proliferative glomerulonephritis, the expression of VEGF and its receptors may be influenced by a variety of immunological factors. In contrast, nephrosis induced by intra-
peritoneal injection of bovine serum albumin (BSA) without unilateral nephrectomy, is not associated with morphological changes in the glomeruli and interstitium, such as mesangial proliferation or fibrosis [19, 20]. Furthermore, BSA is a poor immunogen in rats [21] and until now, no immunological mechanism has been identified in this model [22]. Therefore, this model is appropriate for elucidating the relationship between the development of proteinuria and expression of VEGF.

In the present study, non-radioactive in situ hybridization was performed to examine the expression of VEGF mRNA and its receptors, flt-1 mRNA and KDR/flk-1 mRNA, in BSA-injected rat nephrosis.

Materials and methods

Animals

A total of 40 female Lewis rats (Charles River Breeding Laboratories) were used in the present experiments. The rats were 6–12 weeks old and 180–200 g at the time of the experiment. They were housed individually in metabolic cages and provided with standard rat chow and tap water ad libitum. The cages were designed to allow urine collection throughout the experimental period.

Experimental design

Twenty rats received a daily intraperitoneal injection of 2 g of endotoxin-free BSA (fraction V, Sigma Chemical Co., St Louis, MO) dissolved in saline, under ether anaesthesia. Another 20 rats served as control animals and each were injected daily with an equivalent volume of the vehicle. Treatment continued for various time intervals ranging from 1 to 14 days, followed immediately by euthanasia using a high dose of ether on day 0, 1, 3, 7, or 14. The kidneys were removed, decapsulated, and processed for histological examination. The experimental protocol was approved by the Animal Care Committee of Nagasaki University.

Microscopic examination

A portion of the kidney was fixed and processed manually using standard procedures, embedded in paraffin, and sectioned at 4 μm in thickness. Some sections were stained with periodic acid-Schiff (PAS) for light microscopy. Immunohistochemistry for complement and immuno-globulins were performed in some sections. The sections were embedded in optimal cutting temperature (OCT) compound (Miles Inc. Elkhart, IN) and stored at −80°C until used for in situ hybridization and immunofluorescence studies. Samples of renal tissue sections fixed in buffered 2.5% glutaraldehyde in a 0.1 M phosphate-buffered saline (PBS, pH 7.4) were also examined by electron microscopy.

Probes and labelling for in situ hybridization

We used digoxigenin-labelled oligonucleotide probe. The oligonucleotide probe is often used for in situ hybridization since it has several advantages over other types of probes, as reviewed by Koji and Nakane [23, 24]. Moreover, the expression of specific mRNA is proportional to that of 28S rRNA, which represents the remaining RNA in tissue sections [25]. The sequence of 28S rRNA probe, which was used for examination of retained RNA on tissue, was reported earlier [25]. A 30mer oligonucleotide was designed by a computer software based on the sequence of VEGF [26] and synthesized by a DNA synthesizer (model 391, PCR-MATE EP, Foster City, CA). The sequence of VEGF oligo-DNA corresponded to the base number 202–231 of the cDNA. The probes of receptors for VEGF, rat flt-1, and KDR/flk-1 cDNA were available and cloned as described previously [27]. As a negative control for the cDNA probes, mouse male germ cell-associated kinase (mak) cDNA, which was expressed exclusively in germ cells of the mouse testis [28] was used instead of the cDNA probe for VEGF receptors. Oligonucleotide and cDNA were labelled using a DIG oligonucleotide tailing kit (1175033, Boehringer) according to the standard protocol, respectively.

In situ hybridization

Digoxigenin-labelled in situ hybridization was performed using the method originally developed in our laboratory, as described previously [29–31]. In brief, renal tissue sections were prepared and fixed in 4% paraformaldehyde in PBS, followed by treatment with HCl and digestion with proteinase K (Sigma P4914). After pre-hybridization, the tissues were hybridized overnight in a hybridization buffer containing 1 ng/ml of digoxigenin-labelled probe. After hybridization, the sections were washed with 2 × standard saline citrate buffer (SSC) and hybridized probes were visualized by an immunoperoxidase staining procedure, as described previously [29]. To evaluate the specificity of the technique, a variety of control studies were performed. (i) RNase study; sections were incubated with RNase (Boehringer Mannheim Biochemica) after proteinase K digestion and pre-hybridized and hybridized as described above. (ii) Competition experiment; a large amount of unlabelled oligonucleotide or cDNA was added to the standard hybridization buffer. (iii) Negative controls; in situ hybridization with sense probe and mock cDNA probe, an irrelevant cDNA probe [28], were also performed. The retained RNA in the sections was routinely checked by in situ hybridization for 28S rRNA as described previously [25].

Dot blot hybridization

In order to assess the sensitivity of our in situ hybridization system, we performed dot blot hybridization on a nylon membrane as reported previously [23]. In brief, the serially diluted synthesized sense oligonucleotide was spotted on the membrane and was reacted DIG-labelled antisense and sense oligonucleotides, respectively. To visualize the hybridized probe, the membrane was stained with immunohistochemistry used as in in situ hybridization method. As we described previously [30], as little as 1 pg of sense oligonucleotide was detected with DIG-labelled antisense oligonucleotide, while hybridization with homologous or unrelated oligonucleotide produce no signal (Figure 1a). The intensity of the signal obtained in dot blot hybridization was proportional to the amount of sense oligonucleotide spotted on the membrane (Figure 1b).

Semi-quantitative analysis of VEGF mRNA expression by image analyser-assisted in situ hybridization

In order to assess the results of in situ hybridization semi-quantitatively, we performed image analysis using an
VEGF and its receptors in nephrosis

infiltration of macrophages and proliferation of renal cells, we used antibodies against rat macrophages (ED-1, SEROTEC MCA341, Kidlington, Oxford, UK) and proliferating cell nuclear antigen (PCNA) (Dako M879, Denmark). Sections were reacted with the primary antibody followed by horseradish-peroxidase conjugated antibodies as described previously [29].

Statistical analysis

Data were expressed as mean ± SD. For quantitative analysis, we examined 50 glomeruli in each section and counted the number of cells positive for VEGF mRNA, flt-1 mRNA, and KDR/flk-1 mRNA. We also calculated the ratio of positive cells to the total number of intraglomerular cells in each glomerulus. Differences between data of control and each group of experimental animals (1, 3, 7, or 14 days of treatment) were tested for statistical significance using a one-way analysis of variance (ANOVA) with Scheffé’s F test. A P<0.05 value denoted a significant difference.

Results

Proteinuria

Heavy proteinuria developed in all BSA-treated rats and persisted throughout the study. The level of protein in the urine was maximum in rats injected with BSA for 3 days although its level in rats injected with BSA for 14 days was still higher than control (Figure 2). Saline-treated rats did not develop proteinuria.

Histological examination

Microscopical examination showed no increase of cells and matrices in the glomeruli of BSA-injected rats during the period of observation (1–14 days) (Figure 3a,b). Immunofluorescence studies showed no deposition of immunoglobulins and C3 in both control and BSA-treated nephritic rats (data not shown). These

Immunofluorescence and immunohistochemical staining

Immunofluorescence staining of renal tissues was performed to examine the deposition of complement and immunoglobulin in this model, using monoclonal antibody against C3 (Cappel Products, 55751, Organon Teknika NV) and IgG (Cappel Products, 55756, Organon Teknika NV). Briefly, renal cryostat sections were stained with fluorescein isothiocyanate-conjugated goat antirat C3 and IgG. To examine for
results confirmed that nephrosis is a non-immunological process in BSA-injected rats used in the present study, and are consistent with those demonstrated previously in the protein-overload model [22]. Ultrastructural studies showed a diffuse loss of the foot processes of glomerular epithelial cells along the glomerular basement membrane in nephritic rats treated with BSA for 3 days (Figure 3d) compared with control rats (Figure 3c). The same abnormality was also present in tissues of rats treated for 14 days.

Expression of 28S rRNA and VEGF mRNA

In situ hybridization for 28S rRNA showed many positive cells in the renal tissue, confirming the retained RNA in the section (Figure 4a). Examination of glomeruli in the control animals showed a few cells positive for VEGF mRNA (Figure 4b). In contrast, in BSA-injected rats the number of cells positive for VEGF mRNA in the glomeruli was higher than in control after 3, 7, or 14 days of treatment (Figure 4c,d). VEGF mRNA was localized in cells within the mesangial area (Figure 5a), glomerular visceral epithelial cells (Figure 5b) and parietal epithelial cells (Figure 5c). In the interstitium, no signal for VEGF mRNA was observed in control and BSA-injected rats (Figure 4e,f). Figure 6 shows the ratio of VEGF mRNA-expressing cells in glomeruli in control and nephritic rats. The ratio of cells positive for VEGF mRNA increased after injection of BSA, reaching a maximum value in rats injected with BSA for 3 days, followed by a gradual fall in rats injected with BSA for 7 and 14 days. Figure 7 shows the results of image analysis of glomerular expression of VEGF mRNA. Expression of VEGF mRNA significantly increased after injection of BSA for 3 days compared with control and BSA-injected rat at day 14.

Localization of flt-1 mRNA and KDR/flk-1 mRNA

Weak signals for flt-1 mRNA and KDR/flk-1 mRNA were detected in control glomeruli (Figure 8a). In contrast, a strong expression of mRNAs for both receptors was detected in rats injected with BSA for 3, 7, or 14 days (Figure 8b,c,d). Signals for flt-1 mRNA were localized in cells within the mesangial area (Figure 8e) and glomerular endothelial cells (Figure 8f). The localization of KDR/flk-1 mRNA was similar to that of flt-1 mRNA. The ratio of cells positive for the receptors relative to total number of glomerular cells is shown in Figure 9. The ratio of these cells increased progressively in the nephritic rats reaching a maximum number in rats injected with BSA for 3 days. The ratio of cells positive for flt-1 mRNA was not significantly different from that of KDR/flk-1 mRNA. Glomerular visceral and parietal epithelial cells showed no signal for mRNAs of both receptors in control and nephritic rats. In the interstitium, no expression of mRNA of both receptors was detected (data not shown).

Specificity of in situ hybridization

A number of control studies were performed to confirm the specificity of the signal. In a study using the sense
probe, which was complementary to antisense probe for VEGF mRNA, no signal for VEGF mRNA was observed (Figure 10a,b). Treatment of tissue sections with RNase prior to hybridization eliminated most of the signal for VEGF mRNA and VEGF receptor mRNA (data not shown). As an alternative approach, in a competition study, the addition of a large amount of unlabelled homologous oligonucleotide or cDNA for VEGF receptors to the standard hybridization mixture containing DIG-labelled oligonucleotide probe or cDNA for VEGF receptors, completely abolished the positive signal (Figure 10c; flt-1). In situ hybridization for mak mRNA, negative control for the cDNA probes, showed no positive cells in renal tissues of control and nephritic rats (Figure 10d).

**Immunohistochemical staining for ED-1 and PCNA**

Cells positive for ED-1 and PCNA were sparsely distributed in the glomeruli and interstitium in both control and nephritic rats (Figure 11a,b and Table 1).

**Discussion**

The present study demonstrated that VEGF mRNA and the transcriptional products for its receptors, flt-1 and KDR/flk-1, were expressed in rats with BSA-induced nephritis, as detected by in situ hybridization. Furthermore, the expression of mRNAs of VEGF and its receptors in glomeruli correlated with the severity of proteinuria rather than with the proliferation of endothelial cells in this model.

In situ hybridization using oligonucleotide was previously thought to be not very sensitive compared with other types of probes, such as cDNA and RNA probes. However, the non-radioactive oligonucleotide probe is now sensitive enough to localize specific mRNA at a cellular level [24]. Our non-radioactive method with digoxigenin-labelled oligonucleotide probes has a high resolution, allowing the detection of individual glomerular resident cells positive for various types of specific mRNA [29,30,32]. Applying this method in the present...
study, we have demonstrated that VEGF mRNA was predominantly localized in glomerular visceral epithelial cells and cells in mesangium, and that transcripts of VEGF receptors, flt-1 and KDR/flk-1, were expressed in glomerular endothelial cells and cells in the mesangium.

Several studies have examined the expression of VEGF in renal tissue, but the results were controversial. For example, glomerular visceral epithelial cells and tubular cells in human [6–8,33] and rats [3,4] are known to express VEGF mRNA by radioactive in situ hybridization and the expression of VEGF was reduced in several human glomerular diseases [7,8]. With regard to blood vessels, all studies reported a lack of specific labelling for VEGF in blood vessels of the kidneys [3,4,6–8,33]. All these studies, however, also failed to show mesangial expression of VEGF. On the contrary, Iijima et al. [9,10] showed that cultured mesangial cells synthesize VEGF mRNA. Furthermore, Iruela-Arispe et al. [14] showed that many cells in mesangial area were positive for VEGF mRNA in anti-Thy1 nephritis and postulated that these cells were probably mesangial cells or macrophages. The reasons for the discrepancy between these early results and those of the present study may be due to differences in methodologies, species and type of renal diseases. Previous studies employed radioactive probes for in situ hybridization, while we used digoxigenin-labelled probe in the present experiment. Moreover, VEGF expression has not yet been examined in nephrosis with morphologically normal appearance, like BSA-induced nephritis. Regarding the expression of VEGF, we have to consider the possibility that the expression of transcript and protein may not be parallel, because we did not perform immuno-histochemical study using antibodies to VEGF protein.

A number of cells in mesangial area were positive for VEGF mRNA and its receptors’ mRNAs, suggesting that they were either mesangial cells or monocytes and macrophages. Since macrophages are also able to synthesize VEGF mRNA [34], it is important to examine whether macrophages did not constitute part of intraglomerular cells positive for VEGF mRNA. To test such possibility, we also performed immunohistochemistry for ED-1, a marker for macrophages, and in situ hybridization for VEGF mRNA. By finding a different pattern of staining between cells positive for VEGF mRNA on one hand and ED-1 on the other, as well as a higher number of cells positive for VEGF mRNA than ED-1, we confirmed that glomerular resident cells, may be mesangial cells, pro-
Fig. 8. In situ hybridization of two VEGF receptors, flt-1 (a,b,e,f) and KDR/flk-1 (c,d) mRNA in glomeruli. (a) A few positive cells for flt-1 mRNA in a vascular pole in a glomerulus in a control rat. (b,e) Glomerular expression of flt-1 mRNA (b) and KDR/flk-1 mRNA (e) in treated rats on day 3. The staining pattern of the two receptors is almost the same. (d) KDR/flk-1 mRNA expression in a treated rat on day 14. The expression is less intense compared with that on day 3, but still more intense than in control rats. (e,f) Rats with BSA-induced nephritis on day 3. (*and M indicate capillary lumen and mesangial matrix, respectively). Magnification ×100 (a–d), ×1000 (e,f).

Reduced VEGF mRNA. Further studies are necessary to identify the exact type of renal cells that produce VEGF. In contrast to our model, rats with unilateral nephrectomy before BSA injection showed a high number of macrophages in interstitium and interstitial fibrosis [34,35]. These changes may be associated with hyperfiltration secondary to nephrectomy.

With regard to the expression of VEGF receptors in renal cells, Simon et al. [36] showed by using radioactive in situ hybridization that glomerular cells surrounding the lumina of glomerular capillaries were positive for flt-1 mRNA as well as KDR/flk-1 mRNA in normal human kidney. In the diseased kidney, there is only one report describing the expression of VEGF receptors. Iruela-Arispe et al. [14] using radioactive in situ hybridization found that upregulated KDR/flk-1 mRNA, but not flt-1 mRNA appeared in the capillary wall in anti-Thy1 nephritis, a model of mesangial proliferative glomerulonephritis. Since glomerular endothelial cells stained for PCNA in that model, they postulated that VEGF might be involved in glomerular endothelial proliferation. On the other hand, mesangial proliferation is associated with cytokines and growth factors, such as TGF-β and PDGF [15,16] and these factors increase the expression of VEGF [9,17,18] and flt-1 [17]. Thus, it is possible that the expression of VEGF and its receptors in anti-Thy1 model may be influenced by these factors. In contrast, there is so far no evidence for immunopathologic factors in BSA-injected rats [22]. Considered with our results that few glomerular cells were stained for PCNA and that both VEGF receptors were expressed in our model, it is suggested that VEGF is not associated with endothelial proliferation. The disparity between anti-Thy1 nephritis and our model suggests that VEGF may play a different role in different renal diseases. Moreover, it is possible that two receptors have different roles. In fact, Keyt et al. [37] reported that KDR/flk-1, but not flt-1, was involved in endothelial cell proliferation.

The present study could not establish the exact role...
Table 1. Results of immunohistochemistry for ED-1 and PCNA

<table>
<thead>
<tr>
<th>Days</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>14</th>
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<tbody>
<tr>
<td>ED-1</td>
<td></td>
<td></td>
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<tr>
<td>BSA-injected</td>
<td>2.033±0.964</td>
<td>2.167±1.053</td>
<td>2.067±1.015</td>
<td>2.167±1.020</td>
</tr>
<tr>
<td>Control</td>
<td>2.100±0.712</td>
<td>2.200±0.925</td>
<td>2.033±0.964</td>
<td>2.067±1.143</td>
</tr>
<tr>
<td>PCNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA-injected</td>
<td>1.233±0.817</td>
<td>1.267±0.740</td>
<td>1.233±0.900</td>
<td>1.233±0.817</td>
</tr>
<tr>
<td>Control</td>
<td>1.212±0.825</td>
<td>1.200±0.805</td>
<td>1.197±0.925</td>
<td>1.198±0.964</td>
</tr>
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Note lack of significant increase in cells positive for ED-1 or PCNA in glomeruli of BSA-injected rats compared with control rats.

Fig. 9. Ratio of cells positive for flt-1 and KDR/flk-1 mRNA to the total number of intraglomerular cells. The proportion of positive cells is markedly higher in treated rats at day 3. The ratio of these positive cells gradually decreased but was still significantly higher than in control rats. # and ##, P<0.0001 versus control rats.

of VEGF in BSA-induced nephritis, however, of interest was that alternations of gene expression for VEGF and its receptors in BSA-induced nephritis were proportional to the degree of proteinuria. Previous reports have suggested a possible association between VEGF and proteinuria. Waseda and coworkers [38], demonstrated high serum levels of VEGF in patients with non-insulin dependent diabetes mellitus, with microalbuminaemia and a significant correlation between serum VEGF level and the degree of albuminaemia. Moreover, Iijima et al. [10] showed that injection of VEGF caused proteinuria in rats. In the present study it is suggested that VEGF expression is associated with the development of proteinuria, rather than with proliferation of endothelial cells in rats with BSA-induced nephritis, however, no direct evidence for a causal relationship could be presented. Further studies, including blocking experiments with antibody to VEGF, antagonist and antisense oligonucleotide, are warranted to examine the various roles of VEGF and its receptors in renal diseases.

In conclusion, our results showed that the mRNAs for VEGF and its two receptors, flt-1 and KDR/flk-1, are expressed in glomeruli of rats with BSA-induced nephritis. The presence of a higher proportion of cells

Fig. 10. Control studies examining the specificity of the signal. (a) In situ hybridization using antisense for VEGF mRNA in a treated rat on day 14. Note positive cells in the glomerulus. (b) Stained with sense probe for VEGF mRNA in a section adjacent to that shown in (a); no positive cells. (c) Competition study with unlabelled cDNA for flt-1 mRNA. The signal for flt-1 mRNA is abolished. (d) Negative control study for two VEGF receptors mRNAs. In situ hybridization using mak cDNA, exclusively expressed in testis in a section from a rat injected with BSA for 14 days. No specific staining is seen. Magnification ×100.
positive for the mRNAs in glomeruli was associated with more severe proteinuria. The constitutive expression of VEGF on the mesangium and glomerular epithelial cells and its receptors on the mesangium and glomerular endothelial cells in this BSA-induced nephritis suggest a role in the regulation of glomerular permeability. Our results suggest that VEGF may be involved in the development of proteinuria in the experimental model.

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