

Expression of immunoglobulin E-dependent histamine-releasing factor in idiopathic nephrotic syndrome of childhood

S. Toyabe,* U. Kaneko,* M. Hara and M. Uchiyama

Division of Paediatrics, Department of Homeostatic Regulation and Development, Niigata Graduate School of Medical and Dental Sciences, Niigata University Medical and Dental Hospital, Niigata City, Japan

Accepted for publication 16 June 2005

Correspondence: Dr Shin-ichi Toyabe, Department of Medical Informatics, Niigata University Medical and Dental Hospital, 1-757 Asahimachidori, Niigata City, 951-8520, Japan.

E-mail: address: toyabe@med.niigata-u.ac.jp

*these two authors contributed equally to the study.

Summary

Concanavarin-A (conA)-stimulated peripheral blood mononuclear cells (PBMNC) from patients with idiopathic nephrotic syndrome (INS) produce putative factors that increase vascular permeability. These factors are expressed in the nephrotic phase but are reduced in the convalescent phase. To identify the genes that are expressed only in the nephrotic phase, we performed cDNA subtraction using conA-stimulated PBMNC from three patients with INS. We isolated several gene transcripts in all three subtracted cDNA libraries. Among these genes, IgE-dependent histamine-releasing factor (HRF) was overexpressed in the nephrotic phase not only at the mRNA level but also at the protein level in another 10 patients with INS. Moreover, we found increased secretion of HRF from conA-stimulated PBMNC in the nephrotic phase. The results suggest that HRF is involved in the pathogenesis of idiopathic nephrotic syndrome.

Keywords: vascular permeability factor, concanavalin A, subtraction cloning

Introduction

Idiopathic nephrotic syndrome (INS), also known as minimal change nephrotic syndrome, is the most common cause of childhood nephrotic syndrome and is characterized by massive proteinuria due to increased permeability of the glomerulus [1,2]. Although the aetiology of the disease is unknown [3], several lines of evidence have suggested that putative circulating factors produced by peripheral blood mononuclear cells (PBMNC) play a role in the increased glomerular permeability [4-7]. In adult patients with minimal change nephrotic syndrome, some gene transcripts specifically expressed on PBMNC in nephrotic patients have been identified using molecular biological methods [8,9]. However, the nature of these molecules does not agree with that of the putative permeability factor in INS. In addition, there have been no reports about identification of the permeability factors in children with INS using these methods.

It is well known that concanavalin-A (conA)-stimulated PBMNC of children with INS produce humoral factors that enhance vascular permeability [10,11]. These factors are expressed in the nephrotic phase but are reduced after disappearance of proteinuria. Based on this phenomenon, we tried to identify gene transcripts that are expressed in the nephrotic phase but not in the convalescent phase by using a

polymerase chain reaction (PCR)-assisted cDNA subtraction method. The expansion of PBMNC by conA and amplification of differentially expressed genes by PCR resolved the problem of the small amounts of blood that can be obtained from child donors.

Materials and methods

Patients

Heparinized blood was obtained from 10 patients with INS who fulfilled the criteria of the International Study of Kidney Disease in Children (ISKDC) [12,13]. The patients included two females and eight males, and the average age of the patients at onset of the disease was 6.2 ± 4.3 years. Complication of allergic disorders (medically diagnosed bronchial asthma, allergic rhinitis, allergic conjunctivitis and hay fever) was observed in five patients. High levels of serum IgE (more than 200 IU/ml) were also observed in five patients. The IgE levels were decreased after the disappearance of proteinuria in all patients. Three patients with high IgE levels at onset showed normal levels (< 200 IU/ml) after the disappearance of proteinuria. Blood samples were obtained from the patients in the nephrotic phase before administration of prednisone and in the convalescent phase after cessation of

steroid therapy. Four healthy male volunteers and four patients with bronchial asthma who showed increased levels of serum IgE were used as controls. The mean age of the controls was 6.0 ± 1.2 years. Informed written consent was obtained from parents of all subjects. This study was approved by the local ethics committee.

Cell culture

PBMNC were separated by Ficoll-Hypaque gradient centrifugation. Two 10^6 PBMNC were cultured at 37°C in 5% CO_2 in RPMI1640 medium supplemented with 10% heat-inactivated calf serum with or without $10\text{ }\mu\text{g/ml}$ conA (Sigma, St Louis, Mo, USA) for 48 h. Cultured supernatants were stored in small aliquots at -80°C until use.

Cloning of nephrotic phase-specific transcripts

Total RNA was extracted from the cultured cells using Isogen (Nippon gene, Tokyo, Japan), and poly(A)⁺ RNA was isolated from the total RNA using an Oligotex-dT30 Super mRNA purification kit (Takara, Tokyo, Japan). To obtain cDNA clones that were expressed only in the nephrotic phase, cDNA subtraction was performed using a PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) [14]. The differentially expressed transcripts were subcloned into pGEM-T easy vector (Promega, WI, USA), and the nucleotide sequences were determined using a BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA, USA). Fifty clones from each subtraction cDNA library were randomly selected and sequenced. As a control experiment, subtraction cloning was performed using pooled cDNA samples of conA-stimulated PBMNC obtained from four patients with atopic asthma and samples from four healthy controls.

DNA analysis

The nucleotide sequences of the subtracted clones were analysed by the computer software program GENETYX-Mac (Software Development Co., Tokyo, Japan) using the databases DDBJ (DNA Data Bank in Japan), EMBL (European Molecular Biology Laboratory) and GenBank (National Centre Biology Laboratory). Sequence homology searches were conducted using FASTA and BLAST (basic local alignment search tool) packages on DDBJ.

Reverse transcription PCR

PCR was performed to amplify cDNA for HRF and β -actin using the following primer pairs: 5'-CATGATTATCTAC CGGAC-3' and 5'-TTAACATTTTCCATTCTA-3' for HRF [15], and 5'-CGTGACATCAAAGAGAAGCTG-3' and 5'-GCTCAGGAGGAGCAATGATCT-3' for β -actin.

Preparation of recombinant HRF

The following primer pairs were used to amplify the HRF cDNA: 5' primer, 5'-AAAAGGATCCATGATTATCTACCGG GAC-3'; 3' primer, 5'-AAAAGAATTCTTAACATTTTTC CATTCTA-3'. The resultant PCR product was digested with restriction enzymes Bam HI and EcoRI. The restricted PCR product was cloned into pGEX-4T-2 vector (Amersham Bioscience Co., Piscataway, NJ, USA) and transfected into JM109 competent cells (Promega). Recombinant glutathione S-transferase (GST) fusion protein was cleaved with thrombin, and purification of recombinant HRF was performed using GST Purification Systems (Amersham).

Western blot analysis

The cultured cells were washed twice with phosphate-buffered saline (PBS) and 1×10^6 cells were lysed in NP-40 lysis buffer consisting of 0.5% NP-40, 1% aprotinin, 1 mmol/l diisopropyl fluorophosphate, 5 mmol/l iodoacetamide and 1 mmol/l phenylmethane sulphonyl fluoride. After quantification of protein by the bicinchoninic acid assay (Sigma), the cell lysates ($50\text{ }\mu\text{g/lane}$) and the culture supernatants ($40\text{ }\mu\text{g/lane}$) were analysed by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and separated proteins were transferred to polyvinylidene difluoride (PDVF) membranes (Amersham). The blots were incubated with an affinity-purified anti-HRF antibody generated in a rabbit and then visualized using an ECL system (Amersham) according to the manufacturer's instructions. In the case of the cell lysates, β -actin in the samples was also detected using a rabbit antibody to β -actin (Funakoshi, Tokyo, Japan). The amount of HRF was semiquantified by scanning densitometry and was expressed as a relative value to the fixed amount of recombinant HRF or levels of β -actin blots.

Statistical analysis

Wilcoxon's signed rank test was used to compare the levels of HRF in paired samples. Wilcoxon's rank sum test was used for unpaired comparisons. All tests were accepted as statistically significant if $P < 0.05$.

Results

Identification of nephrotic phase-specific genes

A sensitive PCR-based subtraction method was used to isolate gene transcripts expressed only in the nephrotic phase. We analysed three independent subtraction libraries obtained from three patients, considering the individual differences among patients. Fifty clones from each subtraction library were randomly selected and sequenced. Six kinds of gene transcripts were repeatedly obtained from all three libraries (Table 1). These transcripts were found to encode

Table 1. Identification of nephrotic phase-specific gene transcripts. Fifty subtracted clones were randomly selected from each subtraction cDNA library and sequenced. Genes found in all three cDNA libraries are listed. *Four novel genes showed partial homology to known ribosomal proteins.

Gene name	Maximum length of sequenced nucleotides (bp)	Frequencies of each clone in each cDNA library		
		Patient 1	Patient 2	Patient 3
HRF	753	4/50	5/50	3/50
Thymosin β 4	455	1/50	1/50	2/50
Novel gene*	376	2/50	1/50	1/50
Novel gene*	412	1/50	2/50	1/50
Novel gene*	338	1/50	1/50	2/50
Novel gene*	340	2/50	2/50	1/50

HRF (12 clones), thymosin β 4 (4 clones), and four other novel genes (4–5 clones). The four novel genes showed partial homology to known ribosomal proteins based on the results of homology searches using the programs FASTA and BLAST. The other 117 subtraction clones were not obtained from all three donors. We also performed cDNA subtraction of conA-stimulated PBMNC from the patients with bronchial asthma with reference to that of healthy volunteers. Thirty-four kinds of cDNA clones were isolated from the subtraction library (data not shown). They contained various kinds of cDNA clones, including two clones of interleukin-5, but they did not contain clones encoding HRF.

Nephrotic phase-specific expression of HRF in conA-stimulated PBMNC

Since transcripts for HRF were obtained most frequently in the subtraction libraries, we further studied the expression of HRF in conA-stimulated PBMNC for the patients. RT-PCR analysis of HRF transcripts in the three patients revealed that HRF transcripts were predominately expressed in the nephrotic phase (Fig. 1). We then studied the expression of HRF protein in PBMNC obtained from 10 additional patients with INS. Figure 2 shows representative results of Western blot analysis of the patients' PBMNC probed by an anti-HRF antibody. Again, HRF protein was predominantly expressed in the nephrotic phase. Semiquantification of HRF protein in the conA-stimulated PBMNC obtained from the 10 patients revealed that the expression level of HRF protein was significantly higher in the nephrotic phase than in the convalescent phase ($P < 0.001$, Fig. 2). There was no significant difference between the levels of HRF in patients complicated with allergic diseases and those without allergic diseases or between levels of HRF in patients with and without high levels of serum IgE.

Secretion of HRF from conA-stimulated PBMNC

Since conA-stimulated PBMNC of nephrotic patients have been reported to secrete putative factors that increase

vascular permeability, we then studied the levels of HRF in culture supernatants. Figure 3 shows representative results of Western blot analysis of culture supernatants probed by an anti-HRF antibody. HRF was secreted into culture supernatants of conA-stimulated PBMNC during the nephrotic phase but not during the convalescent phase. Semiquantification of HRF protein in culture supernatants of conA-stimulated PBMNC from 10 patients revealed that the levels of HRF were significantly higher in the nephrotic phase than in the convalescent phase ($P < 0.001$, Fig. 3). There was no significant difference between the levels of HRF in patients complicated with allergic diseases and those without allergic diseases or between the levels of HRF in patients with and without high levels of serum IgE.

Discussion

In the present study, we found for the first time that HRF production by conA-stimulated lymphocytes of children with INS was increased in the nephrotic phase but decreased in the convalescent phase. Our results showed that not only mRNA but also protein of HRF were expressed at higher levels in the nephrotic phase. In addition, a greater amount of HRF was secreted in culture supernatants of conA-stimulated lymphocytes of INS patients in the nephrotic phase than in culture supernatants of conA-stimulated lymphocytes of INS patients in the convalescent phase. Since HRF influences vascular permeability through histamine

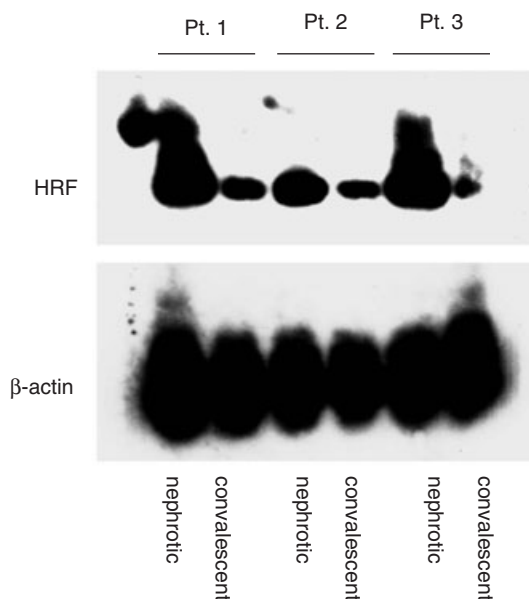


Fig. 1. RT-PCR analysis of HRF transcripts in three nephrotic patients, who were donors for subtraction cDNA libraries. ConA-stimulated PBMNC were obtained from the three patients during both the nephrotic and convalescent phases. RNA was extracted from the cells and RT-PCR analysis for HRF was performed. β -actin was used as an internal standard.

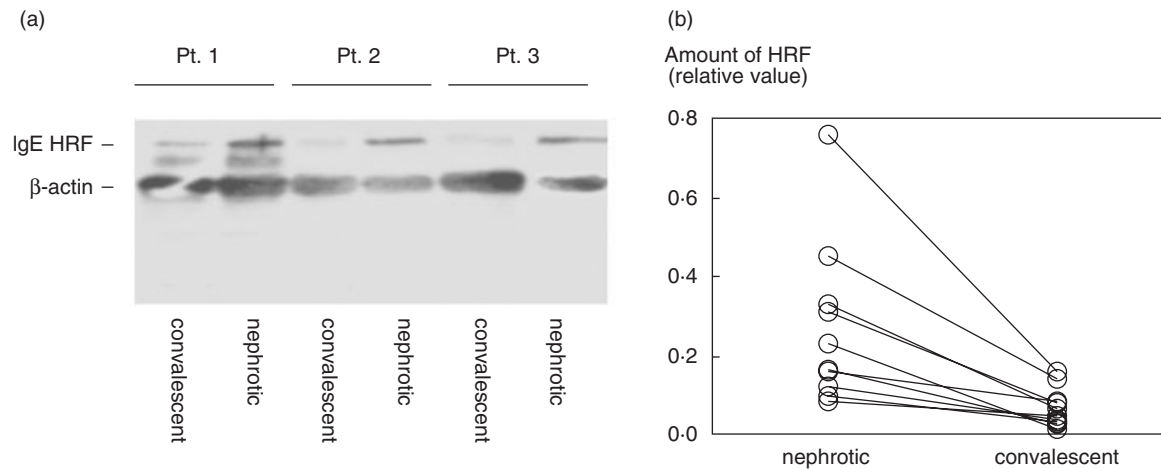


Fig. 2. Expression of HRF protein on conA-stimulated PBMNC from patients with INS. Cell lysates of conA-stimulated PBMNC obtained from three patients, who were donors for subtraction cDNA libraries, were analysed by SDS-PAGE and were subjected to anti-HRF and anti- β -actin blot analysis (a). The amount of HRF was semiquantified by scanning densitometry and was corrected by the level of β -actin. The results for another 10 patients are shown (b).

release [16], HRF might be a candidate for the permeability factors that are produced from activated lymphocytes from patients with INS.

HRF was originally identified as a factor that stimulates release of histamine from basophils in an IgE-dependent fashion and it was later found that HRF has various other bioactivities [17–20]. Increasing vascular permeability is one of the well-known pharmacological functions of histamine [21]. In fact, histidine decarboxylase gene-knockout mice that lack histamine showed no plasma extravasation reaction after a passive cutaneous anaphylaxis (PCA) test, suggesting the importance of histamine in a PCA test [22]. The PCA test is also used as an assay for putative permeability factors in INS [10,11]. Since plasma levels of histamine were reported to be significantly increased in nephrotic patients compared

with those in normal controls [23], the action of the permeability factor that is assayed by the PCA test in INS might be mediated by histamine. This speculation is in agreement with our results showing overexpression of HRF in the nephrotic phase.

Although HRF influences vascular permeability via histamine release, there have been no reports about the effect of HRF on the glomerular capillary permeability. Since there is a marked difference between the structure of the glomerular capillary wall and the structure of walls of other capillaries, it is necessary to investigate the effect of HRF on glomerular capillary permeability. The putative permeability factors in INS have been reported to increase glomerular permeability to albumin [6,24–26], but the molecular basis of the permeability factor has not been identified. Recently, several gene

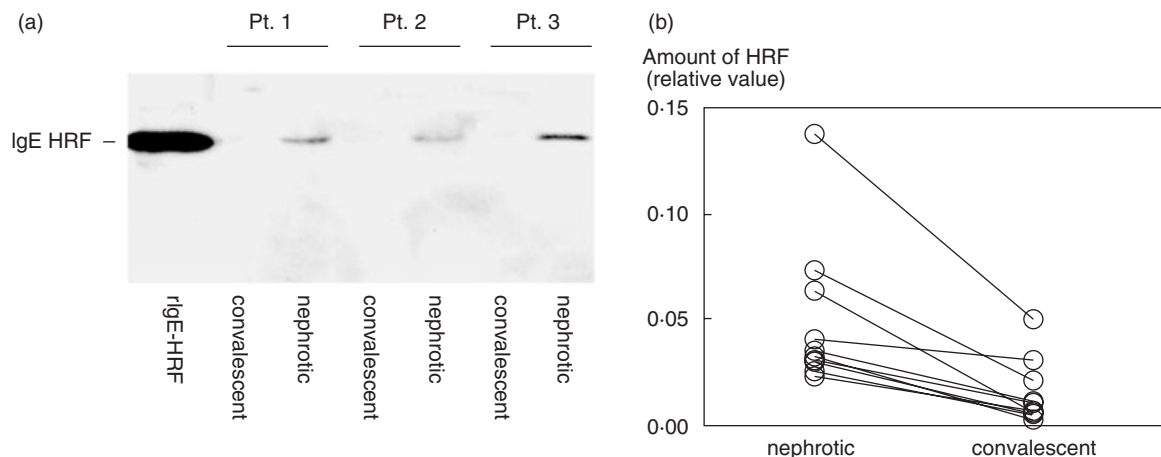


Fig. 3. Expression of HRF protein in culture supernatants of conA-stimulated PBMNC from patients with INS. Culture supernatants of conA-stimulated PBMNC obtained from three patients, who were donors for subtraction cDNA libraries, were analysed by SDS-PAGE and were subjected to anti-HRF blot analysis (a). The amount of HRF was semiquantified by scanning densitometry in another 10 patients (b). The level of HRF was expressed as a relative value to the fixed amount of recombinant HRF.

transcripts that are specifically expressed in the nephrotic phase have been identified in adult patients with minimal change nephrotic syndrome using subtraction cloning [9] and serial analysis of gene expression [8]. Sahali *et al.* [9] identified multiple molecules involved in T cell receptor signalling in adult patients with minimal change nephrotic syndrome by using subtraction cloning, but large proportion of these molecules were not secretory molecules. Interestingly, they isolated three clones of HRF among 84 subtractive transcripts, which is in agreement with our results. Okuyama *et al.* [8] detected the expression of tumour necrosis factor-related apoptosis-induced ligand (TRAIL) in PBMC during the nephrotic phase, but there was no significant increase in serum levels of the soluble form of TRAIL. From these results, it is obvious that the nature of these molecules does not agree with that of the putative permeability factor in INS. Further studies are needed to clarify the role of HRF in the increased glomerular capillary permeability of INS.

References

- Niaudet P. Steroid-sensitive idiopathic nephrotic syndrome in children. In: Avner ED, Harmon WE, Niaudet P, eds. *Pediatric Nephrology*, 5th edn. Philadelphia: Lippincott. Williams & Wilkins, 2003:543–56.
- Haycock G. The child with idiopathic nephrotic syndrome. In: Webb N, Postlethwaite R, eds. *Clinical Paediatric Nephrology*, 3rd edn. Oxford: Oxford University Press, 2003:341–66.
- Shalhoub RJ. Pathogenesis of lipoid nephrosis, a disorder of T-cell function. *Lancet* 1974; **2**:556–60.
- Yoshizawa N, Kusumi Y, Matsumoto K, *et al.* Studies of a glomerular permeability factor in patients with minimal-change nephrotic syndrome. *Nephron* 1989; **51**:370–6.
- Maruyama K, Tomizawa S, Shimabukuro N, Fukuda T, Johshita T, Kuroume T. Effect of supernatants derived from T lymphocyte culture in minimal change nephrotic syndrome on rat kidney capillaries. *Nephron* 1989; **51**:73–6.
- Koyama A, Fujisaki M, Kobayashi M, Igarashi M, Narita M. A glomerular permeability factor produced by human T cell hybridomas. *Kidney Int* 1991; **40**:453–60.
- Garin EH. Circulating mediators of proteinuria in idiopathic minimal lesion nephrotic syndrome. *Pediatr Nephrol* 2000; **14**:872–8.
- Okuyama S, Komatsuda A, Wakui H, Aiba N, Fujishima N, Iwamoto K, Ohtani H, Sawada K. Up-regulation of TRAIL mRNA expression in peripheral blood mononuclear cells from patients with minimal-change nephrotic syndrome. *Nephrol Dial Transplant* 2005; **20**:539–44.
- Sahali D, Pawlak A, Valanciute A, Grimbert P, Lang P, Remy P, Bensman A, Guellaen G. A novel approach to investigation of the pathogenesis of active minimal-change nephrotic syndrome using subtracted cDNA library screening. *J Am Soc Nephrol* 2002; **13**:1238–47.
- Laguerre G, Xheneumont S, Branellec A, Hirbec G, Weil B. A vascular permeability factor elaborated from lymphocytes. I. Demonstration in patients with nephrotic syndrome. *Biomedicine* 1975; **23**:37–40.
- Sobel AT, Branellec AI, Blanc CJ, Laguerre GA. Physicochemical characterization of a vascular permeability factor produced by con A-stimulated human lymphocytes. *J Immunol* 1977; **119**:1230–4.
- ISKDC. Early identification of frequent relapsers among children with minimal change nephrotic syndrome. A report of the International Study of Kidney Disease in Children. *J Pediatr* 1982; **101**:514–8.
- ISKDC. The primary nephrotic syndrome in children. Identification of patients with minimal change nephrotic syndrome from initial response to prednisone. A report of the International Study of Kidney Disease in Children. *J Pediatr* 1981; **98**:561–4.
- Diatchenko L, Lau YF, Campbell AP *et al.* Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci USA* 1996; **93**:6025–30.
- MacDonald SM, Rafnar T, Langdon J, Lichtenstein LM. Molecular identification of an IgE-dependent histamine-releasing factor. *Science* 1995; **269**:688–90.
- Bheekha-Escura R, Chance SR, Langdon JM, MacGlashan DW Jr, MacDonald SM. Pharmacologic regulation of histamine release by the human recombinant histamine-releasing factor. *J Allergy Clin Immunol* 1999; **103**:937–43.
- Schroeder JT, Lichtenstein LM, MacDonald SM. An immunoglobulin E-dependent recombinant histamine-releasing factor induces interleukin-4 secretion from human basophils. *J Exp Med* 1996; **183**:1265–70.
- Schroeder JT, Lichtenstein LM, MacDonald SM. Recombinant histamine-releasing factor enhances IgE-dependent IL-4 and IL-13 secretion by human basophils. *J Immunol* 1997; **159**:447–52.
- Bheekha-Escura R, MacGlashan DW, Langdon JM, MacDonald SM. Human recombinant histamine-releasing factor activates human eosinophils and the eosinophilic cell line, AML14-3D10. *Blood* 2000; **96**:2191–8.
- Yoneda K, Rokutan K, Nakamura Y, Yanagawa H, Kondo-Teshima S, Sone S. Stimulation of human bronchial epithelial cells by IgE-dependent histamine-releasing factor. *Am J Physiol Lung Cell Mol Physiol* 2004; **286**:L174–81.
- Ohtsu H, Watanabe T. New functions of histamine found in histidine decarboxylase gene knockout mice. *Biochem Biophys Res Commun* 2003; **305**:443–7.
- Ohtsu H, Kuramasu A, Tanaka S *et al.* Plasma extravasation induced by dietary supplemented histamine in histamine-free mice. *Eur J Immunol* 2002; **32**:1698–708.
- Gill DS, Fonseca VA, Barradas MA, Balliod R, Moorhead JF, Dandona P. Plasma histamine in patients with chronic renal failure and nephrotic syndrome. *J Clin Pathol* 1991; **44**:243–5.
- Dantal J, Bigot E, Bogers W *et al.* Effect of plasma protein adsorption on protein excretion in kidney-transplant recipients with recurrent nephrotic syndrome. *N Engl J Med* 1994; **330**:7–14.
- Savin VJ, Sharma R, Sharma M *et al.* Circulating factor associated with increased glomerular permeability to albumin in recurrent focal segmental glomerulosclerosis. *N Engl J Med* 1996; **334**:878–83.
- Sharma M, Sharma R, McCarthy ET, Savin VJ. The focal segmental glomerulosclerosis permeability factor: biochemical characteristics and biological effects. *Exp Biol Med* 2004; **229**:85–98.