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Up-regulation of interleukin-4 and CD23/FcεRII in minimal change nephrotic syndrome

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Abstract Although the pathogenesis of childhood minimal change nephrotic syndrome (MCNS) has not been clearly defined, the current hypothesis favors an involvement of T cell dysfunction. The symptom onset and the relapse of MCNS are frequently associated with allergy and increased IgE levels in sera. Since a T cell-derived cytokine interleukin-4 (IL-4) plays a key role in the regulation of IgE production and allergic response, we investigated the role of IL-4 in the pathophysiology of MCNS. Using fluorescence-activated cell scanning we observed a significantly higher expression of CD23, the type II IgE receptor (Fc ε RII), on fresh B cells from active MCNS patients (n=22) compared with age-matched healthy normal controls (n=12). The upregulation of CD23 correlates with greater IL-4 activity in the culture supernatant of MCNS peripheral blood lymphocytes (PBLs) than normal PBLs stimulated by mitogens, as assessed by the CD23-inducing effect of the PBL supernatant on tonsillar B cells. Furthermore, Northern blot and reverse transcription-based polymerase chain reaction analysis have revealed significantly elevated levels of IL-4 mRNAs both in mitogen-stimulated and unstimulated MCNS PBLs, compared with healthy normals or disease controls with other renal disorders. Together these results strongly suggest that the upregulation of IL-4 in T cells may be part of the T cell dysfunction involved in MCNS.

Key words Minimal change nephrotic syndrome · Type II IgE receptor · Interleukin-4 · Interleukin-4 mRNA · Upregulation

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Introduction

Childhood minimal change nephrotic syndrome (MCNS) is a renal disease characterized by heavy proteinuria and hypoalbuminemia with a minute structural change in glomerular membranes involving effacement of foot processes. It has been postulated that a T cell-derived vascular permeability factor may be responsible for the alteration of glomerular permeability by modifying the charge barrier. A variety of lymphokines have been implicated in the pathophysiology of MCNS [1–8]. MCNS is frequently associated with allergic symptoms such as bronchial asthma, allergic rhinitis, urticaria, atopic dermatitis, and an elevated serum IgE level. Relapse is frequently preceded by immunological stimuli, including vaccination, infection, and bee stings, which would stimulate sensitized lymphocytes to produce a number of highly active cytokines [9–11]. Since interleukin-4 (IL-4) has been recognized as a key cytokine regulating IgE production, IgE receptor expression, and thus the allergic response [12–16], we investigated the involvement of IL-4 in the pathogenesis of MCNS. We report a strong association of upregulation of CD23 expression, IL-4 activity, and IL-4 mRNA expression with the active nephrotic state of MCNS.

Materials and methods

Children with MCNS (n=22, 19 males, 3 females, aged 3–12 years) were diagnosed by the age of onset and clinical responsiveness to corticosteroids according to the criteria of the International Study of Kidney Disease in Children. All patients were newly diagnosed. They had no evidence of infection or systemic or renal disease from their history, physical examination, and laboratory findings. Blood samples were obtained during the active nephrotic phase (urinary protein >40 mg/m^2 per hour, serum albumin <2.0 g/dl) before corticosteroid treatment. Control samples were obtained from age-matched healthy normal volunteers (n=12). Patients with other renal diseases, such as focal segmental glomerulosclerosis (FSGS) and IgA nephropathy showing active nephrotic syndrome, served as disease controls (n=5). Tonsils were obtained from the Department of Otolaryngology, Kyung Hee University

Hospital. Statistical analysis was performed using Student's *t*-test. Values are given as the mean plus or minus standard deviation.

Preparation of tonsillar B cells and B cell culture

Fresh human B cells were prepared from normal tonsillar tissue with no signs of inflammation by Ficoll-Hypaque density gradient centrifugation and rosetting twice with 2-aminoethylisothiouronium bromide-treated sheep red blood cells. After removing adherent cells, 97% purity of B cells was obtained, as confirmed by anti-Leu 16 and anti-Leu 4 (Becton Dickinson) staining. Purified B cells were cultured in RPMI media (GIBCO) containing 10% fetal bovine serum (FBS) (Hyclone) 10 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and antibiotics, in a humidified chamber supplied with 5% CO_2.

Preparation of peripheral blood lymphocyte supernatants and CD23 induction assay

Peripheral blood lymphocytes (PBLs) isolated from patients with active MCNS and healthy normal children, using Ficoll-Hypaque, were cultured for 48 h in the presence or absence of mitogens, phorbol myristate acetate (10 ng/ml) plus phytohemagglutinin (5 µg/ml). The culture supernatants were analyzed for CD23-inducing activity to assess the IL-4 content. PBL supernatants (5%–10%) were used to treat the purified tonsillar B cells (1×10^6). Antibody neutralizing assays were conducted using polyclonal neutralizing goat anti-human IL-4 antibodies (R and D systems) mixed with recombinant human IL-4 [17] or T cell supernatants for 30 min at 37°C prior to treatment of B cells. The B cells were then cultured for 24 h, after which membrane CD23 (mCD23) expression was analyzed.

mCD23 measurement

mCD23 expression of fresh and cultured B cells (1×10^6) was analyzed by double antibody staining with fluorescein isothiocyanate-labelled anti-Leu 16 (pan B marker) and phycoerythrin-labelled anti-Leu 20 (CD23) antibodies (Becton Dickinson) in Hanks' balanced salt solution containing 3% FBS and 0.1% sodium azide for 30 min at 4°C, using a fluorescence-activated cell scanner (FACscan, Becton Dickinson). mCD23 positivity among B cells was expressed as $\frac{\text{CD23 positive B cells}}{\text{total B cells}} \times 100\ (\%)$.

IL-4 mRNA analysis

Cytoplasmic RNAs were isolated from fresh PBLs or from those treated with PMA (40 ng/ml) plus ionomycin (400 ng/ml) for 24 h, using the method of Chirgwin et al. [18]. For Northern blot, 10 µg of total RNA from each preparation was separated on a 1% agarose-formaldehyde gel, transferred to nylon membranes (Genescreen Plus, New England Nuclear), and hybridized with a full-length human IL-4 [550-base pair (bp)] cDNA probe [17] labelled with [α-^{32}P]dCTP (Amersham, 3,000 Ci/mmol) at a specific activity of 5×10^8 cpm/µg. RNA concentration was determined by optical density measurement and the amount of RNA loaded onto the gel was confirmed by ethidium bromide staining. A 540-bp *Bam*HI fragment of adenosine phosphoribosyl transferase probe [19] was also used as an internal control to normalize the amount of mRNA in each lane.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed with total RNAs isolated from untreated fresh PBLs of MCNS patients and normals to assess the level of IL-4 transcripts, using human IL-4 primers (5′ primer, 5′ CGGCAACTTTTGACCACGGACACAAGTGCGATA 3′ and 3′ primer, 5′ ACGTACTCTGGTTGGCTTCCTTCACAGGACAG 3′) in the IL-4 Amplimer set (Clontech) and an RT-PCR kit (Boehringer Mannheim), according to the manufacturers' instructions. The products were then analyzed on a 1.5% agarose gel.

Results

Because the expression of CD23 is one of the most-prominent phenotypic changes caused by IL-4, and CD23 is considered a molecular marker of IL-4 activity [12], we first measured the CD23 expression on fresh B cells from MCNS patients and normal subjects. mCD23

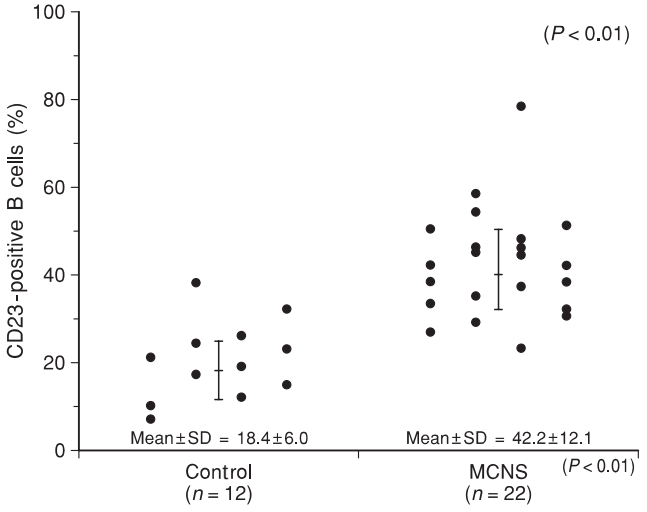

Fig. 1 Levels of membrane CD23 (mCD23) on fresh B cells of minimal change nephrotic syndrome (*MCNS*) patients and normal controls. B cells were isolated from peripheral blood lymphocytes (PBLs) of active MCNS patients and age-matched healthy controls, and mCD23 positivity was determined by FACscan analysis

Table 1 Changes of membrane CD23 (mCD23) levels upon response following steroid therapy of minimal change nephrotic syndrome (MCNS)[a, b]

Patient no.	CD23 levels (% positivity)[c]		Change[d]
	Active stage	Remission stage	
1	58.3	14.4	–
2	48.7	42.3	–
3	47.5	27.7	–
4	45.1	20.0	–
5	43.9	38.2	–
6	37.8	32.0	–
7	28.4	22.5	–
8	24.9	15.3	–
9	25.3	11.1	–
10	22.8	38.4	+
11	37.1	53.0	+

[a] B cells were isolated from peripheral blood lymphocytes of MCNS patients during the active stage prior to steroid therapy and during remission 2 weeks after steroid therapy mCD23 positivity among total B cells was determined by FACscan analysis

[b] Patients were treated with prednisolone 60 mg/m^2 per day in divided doses until 2 weeks after response

[c] All values represent the mean of three independent determinations, and SD ≤10% of the mean

[d] (–) represents decrease and (+) represents increase in CD23 level following steroid therapy

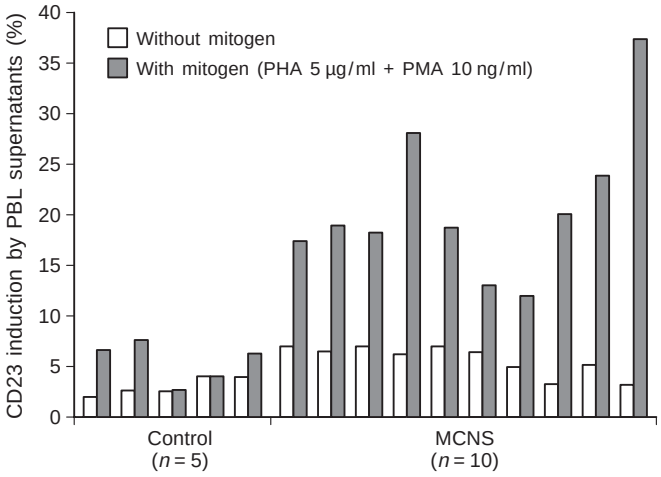

Fig. 2 The CD23-inducing effect of supernatants of PBLs from MCNS patients and normal controls cultured in the absence or presence of mitogens [phytohemagglutinin (*PHA*) and phorbol myristate acetate (*PMA*)]. The PBL supernatants were added to tonsillar B cells at a final concentration of 5%. All values represent a mean of triplicate cultures and SD is less than 10% of mean

expression was significantly higher in active MCNS (mean±SD=42.2%±12.1%) than in normal controls (mean±SD=18.4%±6.0%) (Fig. 1). Among 22 MCNS patients analyzed, 10 had a history of atopy or high serum IgE levels at the time of diagnosis and sampling. However, these patients did not necessarily show higher mCD23 expression than other patients.

The patients shown in Fig. 1 were those analyzed during the active phase of MCNS prior to steroid treatment. Among the 22 patients treated, a follow-up analysis was conducted with steroid-responsive patients (n=11) during the remission stage after 2 weeks off corticosteroids. The CD23 expression was markedly decreased in most of these initial responders (9 of 11), but subsequently increased in 2 patients (Table 1). These results indicate a close association of CD23 with the active nephrotic state. Interestingly, the 2 patients with increased CD23 levels had a relapse soon thereafter, suggesting CD23 as a possible molecular marker of steroid-responsive MCNS.

In order to investigate whether the increased mCD23 expression on MCNS B cells is associated with a high potential of MCNS T cells to produce and secrete IL-4, we assessed the IL-4 activity in the culture supernatant of PBLs stimulated with T cell mitogens. Figure 2 shows that while the CD23-inducing activity of supernatants from untreated PBLs was very low, and basically not different between MCNS patients and controls (mean 5.5% vs. 2.7%), there was a noticeable increase of CD23-inducing activity in the PBL supernatants of MCNS patients compared with normals upon mitogen treatment (mean 20.1% vs. 5.1%). The CD23-inducing activity of these PBL supernatants were effectively inhibited (up to about 70%) by polyclonal anti-IL-4 antibody, indicating that the CD23-inducing effect is primarily due to IL-4 secreted by the activated MCNS PBLs (Fig. 3).

We then analyzed the IL-4 gene expression in these mitogen-stimulated PBLs. When total cellular RNAs, iso-

Fig. 3A, B Blocking effect of anti-interleukin-4 (*IL-4*) antibody on the CD23-inducing activity of culture supernatants of mitogen-treated MCNS PBLs. Purified tonsillar B cells were treated with 200 U/ml IL-4 (**A**) or 5% culture supernatants of unstimulated MCNS PBLs or MCNS PBLs stimulated with PHA and PMA (**B**), in the absense or presense of polyclonal anti-human IL-4 antibodies. mCD23 positivity was then determined. After titration of anti-IL-4 as in **A**, an antibody concentration of 500 ng/ml was used for the neutralizing assay in **B**. Control IgG employed as a negative control had no inhibitory effects. All values represent a mean of triplicate cultures and SD is less than 10% of mean. *NA*, Not applicable

IL-4 (200 U/ml)	α-IL-4	CD23-positive B cells (10 20 30 40 50 60 70 80 90 100)	Inhibition by α-IL-4 (%)
–	–		NA
+	–		NA
+	100 ng/ml		56
+	500 ng/ml		95

A

Patient no.	PBL supernatant mitogen treatment	α-IL-4 (500 ng/ml)	CD23-positive B cells (10 20 30 40)	Inhibition by α-IL-4 (%)
1	–	–		NA
	+	–		NA
	+	+		67
2	–	–		NA
	+	–		NA
	+	+		64
3	–	–		NA
	+	–		NA
	+	+		58

B

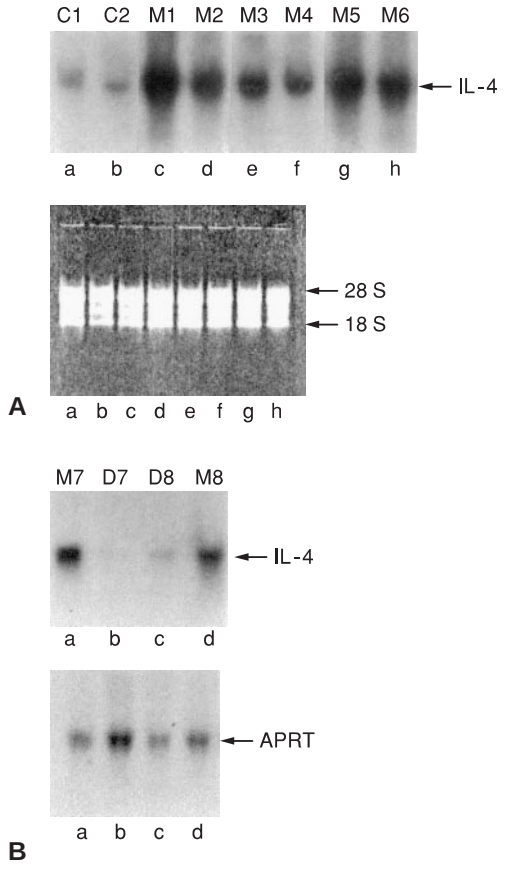

Fig. 4A, B Northern blot analysis of IL-4 mRNA levels. PBLs from MCNS (M1–M8), normal controls (C1 and C2), and disease controls (D7 and D8 for focal segmental glomerulosclerosis and IgA nephropathy, respectively) were stimulated by PMA and ionomycin for 48 h, after which cytoplasmic RNAs were isolated for Northern analysis. The same numbers (e.g., C1 and M1, or C7 and D7) represent the age-matched groups. **A** IL-4 mRNA levels in the mitogen-treated PBLs of MCNS versus controls. *Top*, Northern autoradiogram of IL-4; *bottom*, ethidium bromide-stained RNA gel. **B** IL-4 mRNA levels in the mitogen-treated PBLs of MCNS versus disease controls. *Top*, Northern autoradiogram for IL-4; *bottom*, Northern autoradiogram for adenosine phosphoribosyl transferase (*APRT*). The membrane used for IL-4 analysis was stripped after hybridization and reprobed with an APRT cDNA probe for the internal control

lated from both MCNS PBLs and normal PBLs treated with mitogens, were subjected to Northern hybridization with an IL-4 cDNA probe (Fig. 4A), a strikingly higher expression of IL-4 mRNA (0.65 kilobases) was observed with MCNS (M1–M6) compared with normal samples (C1 and C2). In order to assess whether the increase in IL-4 gene expression is unique to MCNS or common to other nephrotic diseases, PBL samples of FSGS and IgA nephropathy patients were also analyzed. IL-4 mRNA was almost undectectable in these patients (D7 and D8) (Fig. 4B) and comparable to that of normal controls C1 and C2. The mCD23 levels on B cells of these disease controls were also normal, ranging from 18% to 25%.

While these data indicate that MCNS T cells have a greater capacity to induce IL-4 upon mitogenic stimulation, the RNAs isolated from fresh untreated PBLs did not produce any detectable hybridization signal (data not shown). Since it would be important to know whether the MCNS PBLs from the active nephrotic state constitutively express higher levels of IL-4 than normal PBLs, we then conducted RT-PCR, a more-sensitive assay, using fresh untreated PBLs (Fig. 5). By employing two IL-4 primers, which upon PCR produce a 344-bp IL-4 transcript, higher levels of IL-4 were observed in MCNS PBLs (M9–M12) than in age-matched controls (C9–C12).

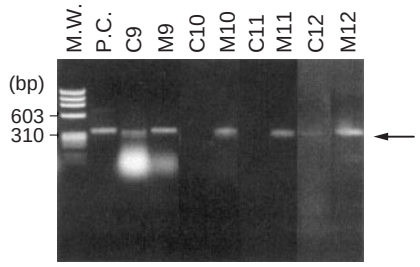

Fig. 5 Reverse transcription-polymerase chain reaction (RT-PCR) analysis of IL-4 transcript in fresh PBLs of MCNS versus controls. Cytoplasmic RNAs, isolated from fresh PBLs of active MCNS patients (M9–M12) and age-matched normal subjects (C9–C12) were used as templates for RT-PCR. The 344-base pair (*bp*) IL-4 transcript is indicated by an *arrow*. *M.W.*, Molecular weight standards; *P.C.*, positive PCR control for IL-4 transcript

Discussion

Since Shalhoub [1] proposed that MCNS represents a generalized disorder of T cells, resulting in production of various humoral factors and cytokines, significant progress has been made in the elucidation of the mechanism and pathogenesis of MCNS. In particular, abnormal cellular immune responses have been described, such as impaired lymphocyte blastogenesis, alteration in lymphocyte subsets, and increased suppressor T cell activities [2]. More recently, a variety of lymphokines have been implicated in MCNS, some of which may alter the glomerular anionic state, inducing a change in membrane permeability. Since active MCNS is frequently associated with allergy, atopy, or high serum IgE levels, we investigated the role of IL-4 in the pathophysiology of MCNS.

Our results clearly show that the expression of CD23, an easily assayable marker of IL-4 activity, is significantly higher on fresh B cells of active MCNS patients than healthy normal controls (Fig. 1). It was interesting to note, among intial steroid responders, a general decrease of CD23 levels in the remission stage following steroid therapy (Table 1). This, together with the observation that the 2 patients with subsequently increased CD23 levels soon relapsed, suggests that CD23 level is an indicator for steroid-responsive MCNS. However, a well-controlled study should be conducted with a larger number of MCNS patients to confirm this.

The increased expression of CD23 on MCNS B cells seems to be due to the greater potential of MCNS T cells

to produce biologically active IL-4, as assessed by CD23-inducing ability of PBL supernatants (Fig. 2). To confirm that the CD23-inducing properties of the PBL supernatant represent IL-4 activity, blocking effects of neutralizing anti-IL-4 antibody were examined; this produced an effective but not complete blockage of CD23-inducing activity (Fig. 3). Although we can not rule out the possibility that the remaining activity is due to other cytokines capable of inducing CD23 expression, such as IL-13 [8, 20, 21], our results indicate that IL-4 constitutes the majority of CD23-inducing activity in the supernatant of MCNS PBLs stimulated by mitogens.

The Northern blot analysis clearly demonstrated that IL-4 gene expression was induced in MCNS PBLs by mitogenic stimuli to a much greater extent than in healthy normals (Fig. 4A) and disease controls with other renal disorders with nephrotic syndrome (Fig. 4B). This suggests that the increased IL-4 production is not a general immune abnormality associated with a variety of nephrotic syndromes, but is characteristic of MCNS. Finally, RT-PCR analysis using RNAs isolated from unstimulated fresh PBLs (Fig. 5) has provided direct evidence that MCNS PBLs not only have a greater capacity to induce the IL-4 gene, but also express a higher level of IL-4 transcript. This would, in effect, result in enhanced IL-4 production and elevated IL-4 activity in the sera of MCNS patients, which in part supports the study of Neuhaus et al. [7].

It appears that the enhanced IL-4 production by MCNS T cells can result in the upregulation of CD23 on B cells. A good correlation was found between the level of mCD23 expression and IL-4 production in the majority of patients analyzed. However, it is not clear whether such increased IL-4 activity is primarily responsible for the elevated serum IgE level often observed in MCNS patients. Other cytokines or factors that synergize or inhibit the action of IL-4 would play a modulatory role in the IgE response in these patients. Interferon-γ which counter-regulates many of the IL-4-induced responses, including IgE production and CD23 (Fc ε RII) expression [15, 16, 22] would be expected to be important in the control of MCNS. We are thus expanding our studies on MCNS to cytokines involved in Th1 and Th2 regulation.

Although the upregulation of CD23 observed in this study may simply reflect the increased IL-4 activity in MCNS patients, an emerging role of CD23 in IgE-dependent allergen presentation and the subsequent activation of IL-4-producing T cells suggests that CD23 functions as a key regulator in the IL-4→IgE/CD23→IL-4 autocrine loop, and thus may be involved in certain allergy-associated disorders [13, 23]. The upregulation of CD23 by IL-4 associated with the active stage of MCNS may in turn further potentiate IL-4 production through T cell activation. While we have shown IL-4 production in MCNS but not other nephrotic diseases, whether IL-4 plays a causative role in the pathogenesis of MCNS remains to be investigated. The initial approach to address this issue would be the analysis of IL-4 in biopsies of MCNS patients.

In conclusion, although considerable work remains to be done to define the pathophysiology of MCNS, our results strongly suggest that MCNS is a T cell disorder involving overexpression of IL-4, and that the upregulation of IL-4 and CD23 may constitute, at least in part, the molecular basis of the T cell abnormality associated with MCNS.

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LITERATURE ABSTRACTS

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Losartan, an angiotensin II type 1 receptor antagonist, lowers hematocrit in posttransplant erythrocytosis

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The mechanism by which angiotensin-converting enzyme inhibitors reduce red cell mass in renal transplant recipients with erythrocytosis is unclear. To examine the role of angiotensin II in this disorder, losartan (a competitive antagonist of the angiotensin II type 1 [AT_1] receptor) was administered to 23 patients with erythrocytosis. Fourteen patients took 25 mg/d for 8 wk; nine others were treated with 50 mg/d for 8 wk. Hematocrit decreased from 0.527±0.027 to 0.487±0.045 after 8 wk (P<0.01) – by at least 0.04 in 19 patients. Decrement in hematocrit in the initial 8 wk of therapy was significantly greater in patients administered 50 mg/d than in patients on 25 mg/d. Twelve of 14 patients initially treated with 25 mg/d showed a small change in hematocrit; the dose was increased to 50 mg/d for 8 more wk. Hematocrit decreased from 0.528±0.030 before losartan treatment to 0.483±0.055 after 16 wk (P<0.01). After therapy, serum erythropoietin significantly decreased in eight patients with elevated baseline levels, but not in 15 patients with normal baseline levels; however, hematocrit significantly decreased in both groups. Losartan was withdrawn in 16 patients; hematocrit increased from 0.440±0.057 to 0.495±0.049 after 8.9±7.5 wk (P<0.001), without change in serum erythropoietin. Thus, specific blockade of AT_1 receptors inhibited erythropoiesis, suggesting a pathogenic role for angiotensin II in posttransplant erythrocytosis.

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Acute changes in urine protein excretion may predict chronic ifosfamide nephrotoxicity: a preliminary observation

Cancer Chemother Pharmacol (1998) 41:413–416

Purpose To evaluate proteinuria occurring early after ifosfamide therapy and to assess the use of changes in proteinuria in the prediction of severe chronic nephrotoxicity.
Methods One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to characterize urine protein excretion in 12 children with solid tumours before and after the first course of ifosfamide treatment, and in 24 healthy children. Chronic nephrotoxicity was evaluated at 6 months after ifosfamide treatment and graded as none, mild, moderate or severe.
Results Urine from healthy children and from 10 of 12 patients before ifosfamide therapy showed a protein band with a molecular weight (95.4 kDa) corresponding to that of Tamm-Horsfall protein but no lower molecular weight proteins. After the first course of ifosfamide this 95.4-kDa protein was lost in six of ten patients with a concomitant appearance of a low molecular weight proteinuria (<70 kDa) in eight. Tamm-Horsfall protein was lost in two of five patients who subsequently developed no or mild nephrotoxicity and in four of five patients who subsequently developed moderate or severe nephrotoxicity.
Conclusions Early subclinical changes in urine protein excretion after ifosfamide, manifested by a loss of Tamm-Horsfall protein excretion, may be predictive of subsequent chronic nephrotoxicity.