Journal of Investigative Medicine 56(8):997–1003, 2008

ORIGINAL ARTICLE

Effect of a Single Intravenous Immunoglobulin Infusion on Neutrophil Gelatinase-Associated Lipocalin Levels in Proteinuric Patients With Normal Renal Function

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ABSTRACT

The aim of the present study was to evaluate levels of neutrophil gelatinase-associated lipocalin (NGAL), a stress protein increased after renal and systemic stimuli, in a cohort of 15 patients with severe proteinuria secondary to idiopathic membranous nephropathy and conserved renal function. Neutrophil gelatinase-associated lipocalin levels and the fractional excretion of this protein were higher in patients than in healthy controls. Furthermore, a close correlation was found between serum NGAL and urinary (uNGAL) ($r = 0.81$; $P < 0.01$) and between uNGAL and daily proteinuria ($r = 0.44$; $P < 0.03$). One hour after infusion of a single high-dose bolus of intravenous immunoglobulin (0.4 g/kg), a new and promising therapy for several kidney diseases, a marked reduction was found in NGAL levels (serum NGAL 194.1 ± 121 vs 370.1 ± 180.5 ng/mL, $P < 0.05$; urinary NGAL 153.3 ± 108.6 vs 502.2 ± 293.4 ng/mL, $P < 0.03$); this was maintained 24 hours after the treatment. The findings made suggest that the NGAL balance is altered in patients with severe proteinuria who have not yet developed overt chronic renal failure, thus confirming the potential use of this protein as an early biomarker of kidney damage preceding the increase in serum creatinine levels. Furthermore, also extra-renal cells (neutrophils, endothelium) may hyper-release NGAL, expressing systemic stress related to severe proteinuria. This would explain the impressive decrease occurring in NGAL values after intravenous immunoglobulin infusion, thus providing further evidence of the antiinflammatory properties of this particular therapeutic approach and indicating the possible value of NGAL measurement in monitoring the efficacy of treatment of renal diseases.

Key Words: neutrophil gelatinase-associated lipocalin, proteinuria, nephrotic syndrome, membranous glomerulonephritis, intravenous immunoglobulin

INTRODUCTION

The etiopathogenic treatment of severe proteinuric conditions associated with idiopathic glomerulopathies is a controversial issue. Steroids and immunosuppressive drugs, which have long been used in the management of these diseases, often incur a high risk of adverse effects, and their benefits are uncertain[1]; nor does exclusively symptomatic treatment appear to reduce the progression of renal damage.[2]

In the search for alternative therapeutic options, various studies have independently demonstrated the efficacy and safety of the chronic intravenous administration of human immunoglobulins (intravenous immunoglobulin, IVIg) in providing stable remission of proteinuria without having significant adverse effects on patients with severe nephrotic syndrome associated with membranous nephropathy.[3–5] This type of approach, initially used for treating several types of immunological diseases, including autoimmune neuropathy, thrombotic purpura, Kawasaki disease and Guillan-Barrè syndrome,[6] has recently been used for an increasing number of kidney diseases (ie, IgA nephropathy, focal segmentary glomerulosclerosis, renal vasculitis, chronic nephropathy after kidney transplantation), and the promising results obtained in this setting indicate interesting possibilities for its future use.[7]

However, whatever treatment chosen for these patients, a significant reduction in daily proteinuria, or even its complete remission, is a fundamental goal in preventing the progression of renal disease, especially if an overt chronic renal failure (decrease in glomerular filtration rate, GFR) has not yet developed.

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In fact, as well as being a sign of renal impairment, persistent proteinuria is, in itself, also a potential cause of damage, especially to the renal tubule, as the sustained transit of plasmatic proteins within the tubular lumen is a source of injury to epithelial cells because of the activation of complement cascade. This last condition triggers tubular inflammation, leading to tubulo-interstitial fibrosis, which ultimately signals irreversible renal impairment leading to chronic renal failure.[8]

In recent years, several proteic and nonproteic factors released by injured epithelial cell have been proposed as biomarkers of tubular impairment; recently reported findings suggest that neutrophil gelatinase-associated lipocalin (NGAL), a small protein initially discovered in activated neutrophils, is one of the most interesting and promising indicators of acute renal impairment.

The dramatic increase in NGAL levels occurring immediately after the induction of experimental ischemic, infective, or toxic damage in mice precedes the increase in serum creatinine levels[9]; in human models, different authors have demonstrated that NGAL measurement is an extremely useful early predictor of acute renal failure after conditions or treatments that are potentially harmful to the kidney, such as cardiac surgery,[10,11] coronary angiography,[12] diarrhea-associated hemolytic uremic syndrome[13] and renal transplantation[14]; this tool allows the timely planning of adequate therapeutic measures. In addition, findings reported in recent literature suggest that this protein may also play a key role in the pathophysiology of several pathological conditions associated with chronic renal failure, including autosomal dominant polycystic kidney disease,[15] pediatric vasculitis,[16] and proteinuric glomerulonephritis,[17,18] in which NGAL values are closely correlated with the common index of residual renal function (GFR, serum creatinine).

The aim of the present study was therefore to evaluate serum and urinary NGAL levels in a small cohort of patients affected by sustained proteinuria associated with idiopathic membranous glomerulonephritis without renal function impairment. The subjects, chosen for the initiation of long-term IVIg pulse therapy rather than routine pharmacological treatment, also underwent analysis of NGAL values after the first bolus of IVIg in order to assess whether this treatment may influence the systemic balance of this biomarker.

■ MATERIAL AND METHODS

Patients Profile

The study cohort consisted of 15 patients (8 males, 7 females; mean age 55 ± 16) with stable severe proteinuria (3.95 ± 1.99 g/24 hours) associated with idiopathic membranous glomerulonephritis. The diagnosis was confirmed on the basis of findings made in renal biopsies, obtained on average 3.1 months (range 2.6–8.4) before starting the first bolus of IVIg. Before initiating IVIg treatment, all patients received diuretics and human albumin in order to treat edema and hypoproteinemia associated with severe proteinuria. No other treatment with, for example, steroids or immunosuppressors was prescribed. Any antihypertensive treatment was discontinued for 1 week before the start of the study. Exclusion criteria were cancer, infection, vasculitis, or leucocyte-count alterations. All the subjects had a normal renal function, with a GFR of 90.9 ± 9.5 mL/min (assessed using the Cockcroft-Gault formula) and serum creatinine values of 0.83 ± 0.55 mg/dL. The treatment protocol and the study design were approved by the local ethics committee, and fully informed consent was obtained from all patients.

Control Group

The control group consisted of 10 healthy volunteers (6 males ,4 females; mean age 47 ± 12) without a history of arterial hypertension, diabetes or neoplastic, cardiovascular, renal, pulmonary, or endocrinal diseases. None of the subjects were on medical treatment, and all had mean serum creatinine levels of 0.71 ± 0.42 mg/dL and a mean GFR (creatinine clearance assessed using the Cockcroft-Gault formula) of 100.7 ± 16.3 mL/min. All control subjects gave their fully informed approval to take part in the study.

Complete data from patients and controls are summarized in Table 1.

IVIg Treatment

Human polyclonal immunoglobulin was administered to each patient as a single intravenous bolus in a dose of 0.4 g/kg (Sandoglobulin; Novartis, Nürnberg, Germany, containing 60 mg/mL polyvalent immunoglobulins). Immunoglobulin infusion, achieved gradually, was completed within 8 hours in all cases and any significant collateral effect was observed. All the patients were required to take chronic long-term pulse IVIg therapy until stable clinical remission of proteinuria, as described elsewhere,[3] was achieved. The data reported in the present study, however, refer only to the effects of the first dose administered (starting bolus).

Collection of Blood and Urine

Blood and urine samples were obtained before the first bolus of IVIg therapy, immediately after, and 1 hour and 24 hours after the first infusion. The blood samples were placed immediately in chilled vacutainer tubes containing potassium ethylenediaminetetracetate, and plasma was promptly separated in a refrigerated centrifuge; samples were stored at −80°C until assayed.

TABLE 1. Main Characteristics of Patients and Controls

	Patients (n = 15)	*Controls (n = 10)*
Age, y	55 ± 16	47 ± 12
Sex (M/F)	8/7	6/4
Time from diagnosis, months	3.1 (2.6–8.4)	
Systolic blood pressure, mm Hg	131 ± 9	119 ± 11
Diastolic blood pressure, mm Hg	83 ± 8	79 ± 4
Weight, kg	74 ± 6	79 ± 5
Body mass index	28.3 ± 5	29.1 ± 4
White blood cell count ($\times 10^6$/mL)	7.9 ± 2	6.5 ± 1.5
Serum creatinine, mg/dL	0.83 ± 0.55	0.71 ± 0.42
Urinary creatinine, mg/dL	75.7 ± 35.5	68.1 ± 12.3
GFR (Cockcroft/Gault), mL/min	90.9 ± 9.5	100.7 ± 16.3
Total proteins, g/dL	4.90 ± 2.56	7.22 ± 2.32
Serum albumin, g/dL	2.96 ± 1.22	4.48 ± 1.38
Cholesterol level, mg/dL	339.8 ± 133.4	199.4 ± 99.1
Triglycerides, mg/dL	195.6 ± 88.8	133 ± 69.6
Proteinuria, g/d	3.95 ± 1.99	0.09 ± 0.05
Serum NGAL, ng/mL	370.1 ± 180.5*†‡	56.2 ± 30.6
Urinary NGAL, ng/mL	502.2 ± 293.4§¶	7.3 ± 6.1
FeNGAL, %	1.95 (1.02–2.89)‖	0.12 (0.06–0.42)

*$P < 0.01$ vs controls.
†Correlation with urinary NGAL ($r = 0.81$, $P < 0.01$).
‡Correlation with proteinuria ($r = 0.19$; $P < 0.05$).
§$P < 0.001$ versus controls.
¶Correlation with proteinuria ($r = 0.44$, $P < 0.03$).
‖$P < 0.05$ versus controls.

Ten milliliters of fresh urine was mixed with 1 mL of 10 mM tris buffer, pH 8.6 with 0.05% Tween 20 and 0.01% of NaN_3 containing protease inhibitors (10 mM benzamidine, 10 mM aminocaproic acid, 20 mM ethylenediaminetetracetate and aprotinin). The mixture was then centrifuged at 3000 rpm for 8 minutes and stored at −80°C until assayed. All urine and blood specimens were used for the study within 3 months of their collection.

NGAL Enzyme-Linked Immunosorbent Assay

Neutrophil gelatinase-associated lipocalin was measured in the blood and urine using enzyme-linked immunosorbent assay commercial available kit (Antibody Shop, Gentofte, Denmark) according to the manufacturer's instructions. All specimens were frequently diluted so as to obtain the concentration required for optimal density according to the enzyme-linked immunosorbent assay kit instructions. Enzymatic reactions were quantified in an automatic microplate photometer. All measurements were made blind in triplicate. Neutrophil gelatinase-associated lipocalin levels were expressed as ng/mL.

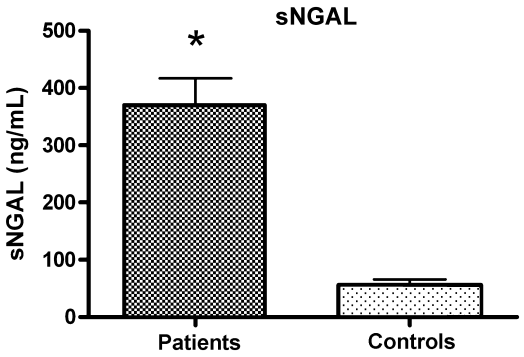

FIGURE 1. Difference between controls and proteinuric subjects for serum NGAL levels (*$P < 0.01$).

Statistical Analysis

The statistical analysis of data was made using MedCalc (version 9.3.0) software (MedCalc Software, Mariakerke, Belgium) and the GraphPad Prism (version 4.0) package (GraphPad Software Inc, San Diego, CA). Data were expressed as mean ± SD and median (range) where appropriate. An unpaired 2-tailed *t* test was used to compare data from the 2 groups, and Pearson correlation coefficient was used to test correlations between variables. Analysis of variance was made using 1-way analysis of variance (ANOVA) followed by Fisher exact test. A *P* value less than 0.05 was considered significant.

■ RESULTS

Differences Between Proteinuric Patients and Control Subjects NGAL Levels

Healthy subjects had serum NGAL levels (sNGAL) of 56.2 ± 30.6 ng/mL, and urinary NGAL levels (uNGAL) were 7.3 ± 6.1 ng/mL, these values fall within the normal range specified in literature.

On the contrary, in proteinuric patients, sNGAL and uNGAL values were markedly higher than in controls,

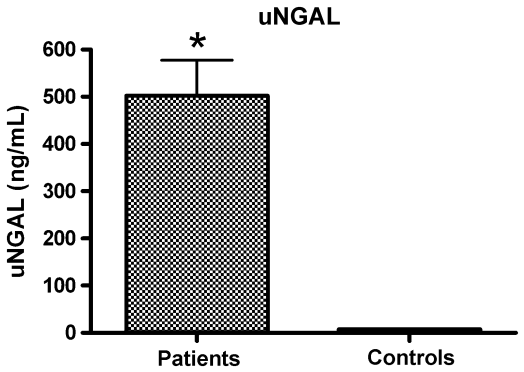

FIGURE 2. Difference between controls and proteinuric subjects for urinary NGAL levels (*$P < 0.001$).

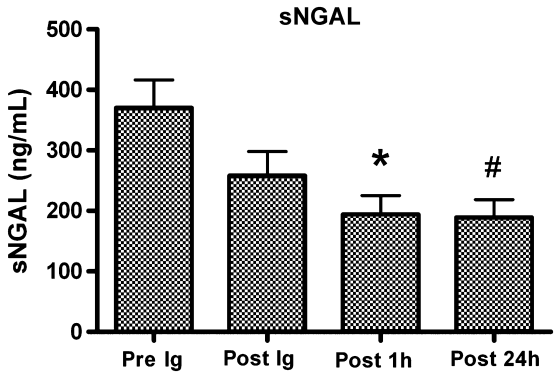

FIGURE 3. Variation in serum NGAL levels from baseline after IVIg treatment (*$P < 0.05$, #P not significant vs post 1 h).

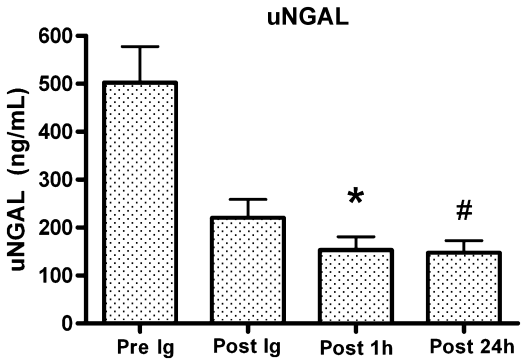

FIGURE 4. Variation in urinary NGAL levels from baseline after IVIg treatment (*$P < 0.03$, #P not significant vs post 1 h).

being 370.1 ± 180.5 ng/mL ($P < 0.01$; Fig. 1) and 502.2 ± 293.4 ng/mL ($P < 0.001$; Fig. 2), respectively.

Fractional Excretion Rate of NGAL in Proteinuric Patients and Controls

In order to evaluate possible sources of urinary NGAL under different conditions, a fractional excretion rate of this protein (FeNGAL) was calculated in healthy and proteinuric subjects, using the following formula: FeNGAL (%) = (uNGAL/sNGAL) × (serum Creatinine/urinary Creatinine) × 100.

The median FeNGAL level was significantly higher in proteinuric patients (1.95%; range, 1.02–2.89) than in controls (0.12%; range, 0.06–0.42) ($P < 0.05$).

Statistical Analysis of Baseline NGAL Levels in Proteinuric Patients

In proteinuric patients, a very close correlation was found between sNGAL and uNGAL levels ($r = 0.81$; $P < 0.01$); furthermore, uNGAL levels were also found to be directly correlated with the extent of daily urinary protein loss ($r = 0.44$; $P < 0.03$). On the contrary, a weak correlation was found between sNGAL and proteinuria ($r = 0.19$; $P < 0.05$). Interestingly, in contrast findings reported in recent literature,[15–18] no correlation was identified between both serum and urinary NGAL levels and GFR or serum creatinine (data not reported).

Effects of Immunoglobulin Treatment on NGAL Levels and Renal Parameters

The infusion of a single, high-dose bolus of IVIg induced an impressive decrease in NGAL levels from baseline. This reduction, manifested immediately after ending the administration of IVIg, attained statistical significance after 1 hour for both sNGAL (194.1 ± 121 vs 370.1 ± 180.5 ng/mL, $P < 0.05$) and uNGAL levels (153.3 ± 108.6 vs 502.2 ± 293.4 ng/mL, $P < 0.03$), and was constant, being found as late as 24 hours after completion of treatment (Figs. 3 and 4). This trend, observed in each patient, was shown to be independent from the individual duration of intravenous infusion.

After completion of IVIg, the fractional excretion rate of NGAL was slightly reduced with respect to baseline (median 1.74%, range 1.01–2.65), although not to a statistically significant extent ($P = 0.25$). Interestingly, the correlation between serum and urinary NGAL levels observed before IVIg infusion was also found after treatment, although slightly lowered ($r = 0.70$; $P < 0.05$). On the contrary, no significant deviation was observed with respect to baseline values for the other parameters (GFR, serum and urinary creatinine, and protein excretion rate) (Table 2).

To exclude intravenous liquid infusion as a potential confounding factor in NGAL variations, 5 control patients were given simple saline solution in a volume (~4 L) equivalent to that of IVIg. This placebo did not

TABLE 2. Effect of a Single IVIg Bolus

	Baseline	*After IVIg*	*After 1 hour*	*After 24 hours*
Serum NGAL, ng/mL	370.1 ± 180.5	258.5 ± 155.4	194.1 ± 121*	189.6 ± 115.3
Urinary NGAL, ng/mL	502.2 ± 293.4	220.3 ± 151.5	153.3 ± 108.6†‡	147.2 ± 99.1‡
FeNGAL, %	1.95 (1.02–2.89)	—	1.74 (1.01–2.65)	1.81 (1.05–2.77)
Serum creatinine, mg/dL	0.83 ± 0.55	—	0.79 ± 0.39	0.81 ± 0.42
Urinary creatinine, mg/dL	75.7 ± 35.5	—	69.9 ± 39.8	71.5 ± 36.6
Urinary protein/creatinine ratio, mg/mg	49.3 ± 19.5	—	47.8 ± 18.8	48.6 ± 20

*$P < 0.05$ versus baseline.
†$P < 0.03$ versus baseline.
‡Correlation with serum NGAL; $r = 0.70$; $P < 0.05$.

modify serum and urinary NGAL or FeNGAL levels with respect to baseline.

■ DISCUSSION

The findings made in the present study yield 2 major points of interest.

First, it was clearly evidenced that in patients affected by severe proteinuria associated with membranous glomerulonephritis, both serum and urinary NGAL levels are markedly higher than in healthy controls; this observation is in agreement with those made in previous studies, which report increased NGAL values in subjects with persistent proteinuria with chronic renal failure,[16–18] suggesting that this condition influences the pathophysiological balance of the protein.

In the present study, however, only proteinuric patients with normal values of GFR and serum creatinine (an important pre-requisite for IVIg therapy) were selected. In this setting, therefore, any confounding effect on NGAL balance due to chronic renal failure was obviated. In fact, other authors have shown that, irrespective of the type of disease and any proteinuria, all subjects with a manifest decrease in GFR have elevated NGAL values that are closely related to residual renal function.[15–18]

In normal conditions, circulating neutrophils are the main source of sNGAL; as demonstrated in vivo, the most important biological function of the protein is the inhibition of bacterial growth.[19] However, it has been reported that sNGAL levels can be increased in patients with some systemic diseases not necessarily associated with infective processes, thus confirming that many other tissues can produce and release NGAL as an acute phase factor signaling sustained injury. For example, NGAL is markedly induced in the course of inflammation involving the endothelium and the epithelia of the intestine, skin, and distal and proximal airways[20,21]; it is also hyper-expressed in atherosclerotic plaques and tissues from myocardial infarction,[22] probably because of the direct involvement of this protein in vessel remodeling mediated by the endothelium and intima.[23] In the renal tubule, NGAL is released in large quantities within a short time after harmful stimuli.[9]

In our patients, overt renal failure or any other systemic process that might have influenced sNGAL values was ruled out. It was therefore reasonable to assume that the increase found in the levels of the protein indicated early renal injury due to severe and persistent proteinuria. However, although this explanation is plausible, other extra-renal tissues may have contributed to the markedly increased sNGAL levels. It has, for example, been observed that circulating neutrophils from patients with severe nephrotic syndrome present an increased cytokine release and antioxidative function, showing a higher selenium content and an enhanced glutathione peroxidase activity with respect to those obtained from healthy age-matched subjects,[24] probably as the consequence of exposition to a systemic insulting oxidative stress associated with this pathological condition.[25] In fact, the so-called oxidative environment, which indicates severe proteinuria, appears to influence the entire immune system: in these conditions, a marked increase has been found in the production and release of some inflammatory chemokines, such as TNF-α, IL-2, and IL-4,[26,27] which are thus increased in plasma as well as fibrinogen and IL-6.[28] Moreover, it has been reported that endothelial function is significantly impaired, thus probably contributing to the increased cardiovascular risk typical of these patients.[28]

The idea of a significant systemic contribution to increased sNGAL values would further explain the lack of a correlation between sNGAL and renal function observed in the present study, in apparent contradiction with data previously reported in literature,[15–18] indicating that in proteinuric patients with conserved kidney function, renal cells may not be the only cause of the increase found in sNGAL levels.

The marked increase in uNGAL levels in our patients raises the question of the significance of increased urinary excretion of this protein. One explanation might be that it depends on an increased leakage of circulating NGAL through the damaged glomeruli. If this were so, a large quota of total uNGAL would be nothing but circulating passively lost NGAL, as occurs with other plasmatic proteins. This hypothesis, supported by the close correlation found between sNGAL and uNGAL ($r = 0.81$), is further borne out by the finding that uNGAL levels were also closely correlated with the extent of daily proteinuria, as described elsewhere.[18] This does not rule out the possibility that tubular cells may at least in part contribute to increased uNGAL levels through active production and urinary release, in a manner similar to that observed during acute tubular injuries, as a defense mechanism against intracellular oxidative stress induced by persistent proteinuria. Furthermore, it has already been demonstrated that the kidney tubule effectively responds to a sustained proteic-overload through the copious release of other "tubular stress" proteins, such as the kidney injury molecule-1, whose levels are consequently increased in urinary fluids.[29]

Furthermore, the finding that proteinuric patients presented FeNGAL levels that were significantly higher than in controls, in all cases above the unity, appears to support the starting hypothesis that tubular dysfunction is involved in determining this condition. This report, as well as the close correlation between uNGAL and daily proteinuria, thus confirms that NGAL may be used as an early biomarker reflecting the presence of

kidney damage in proteinuric patients with conserved renal function, even if serum creatinine and GFR still remain in the normal ranges.

The second aim of the study was to evaluate, in these patients, the effect of a single high-dose bolus of IVIg in order to ascertain whether this treatment may somehow alter the systemic balance of NGAL. The findings made confirm our starting premise, showing a marked reduction in both serum and urinary NGAL values immediately after IVIg and 24 hours after the end of treatment. On the contrary, no change in the overall protein excretion rate was noticed from baseline; the influence of IVIg bolus was thus restricted to NGAL balance.

Furthermore, no significant modification was observed when simple saline solution was administered in a volume equivalent to that of IVIg infusion; this appears to rule out that the reduction in NGAL values is due to an aspecific "dilution" secondary to intravenous infusion rather than being directly due to a pharmacological effect.

In view of the fact that increased baseline sNGAL values may in part represent the expression of a systemic immune dysregulation associated with severe proteinuria, as occurs with other cytokines, our reports are in agreement with those reported by other authors, who previously attributed several antiinflammatory properties to IVIg, thus explaining their efficacy in the treatment of several autoimmune disorders. For example, IVIg has been attributed with the ability to modulate complement activation products, contrasting idiotypic antibodies, saturating Fc receptors on macrophages, and suppressing the release of various inflammatory mediators, including cytokines and metalloproteinases from these cells.[30] In children with Kawasaki disease, the infusion of high doses of IVIg causes a marked decrease in serum IL-6[31] and IL-10,[32] also reducing monocyte chemoattractant protein-1 production and release from peripheral mononuclear cells and polymorphonuclear leukocytes,[33] whereas the same treatment also has a stabilizing effect on the inflamed endothelium, blocking the expression of several chemokines, such as VCAM-1, IL-1beta, monocyte chemoattractant protein-1, IL-6, tumor necrosis factor-α, and vascular endothelial growth factor,[34,35] and stimulating its regeneration through an increase in peripheral circulating progenitor cells, as previously demonstrated in patients with systemic lupus erythematosus.[36] This "antiinflammatory theory" would, moreover, explain why IVIg infusion also induced a significant reduction in uNGAL levels.

As stated previously, if patients have a significant increase of uNGAL consequent to a passive loss through the damaged glomerular barrier, as occurs for other plasmatic proteins, the IVIg-induced reduction in sNGAL levels would also lead to a subsequent decrease in uNGAL values. This hypothesis is strongly supported by the correlation found between sNGAL and uNGAL, already reported at baseline, and which was persistent, being found 24 hours after the end of the infusion; there were no significant changes found in FeNGAL with respect to starting values.

In conclusion, the findings made in the present study clearly indicate that severe proteinuria markedly alters the NGAL balance in patients with severe proteinuria associated with idiopathic membranous glomerulonephritis, even in the absence of overt chronic renal failure.

Furthermore, a single high-dose bolus of IVIg can induce a dramatic decrease in NGAL levels, probably as the consequence of diffuse, systemic immunemodulation, as observed in other cytochines. The concept of that IVIg has an overall systemic effect, does not, however, rule out that the decrease in NGAL values may also depend in part on the direct beneficial effect on the renal tubular epithelium of immunoglobulins, with a consequent reduction in proteinuria-induced oxidative stress. This hypothesis merits further investigation. If confirmed, the reduction in NGAL would further validate the efficacy and safety of the administration of IVIg in patients with renal pathology. Further studies are therefore eagerly awaited to ascertain whether NGAL measurement may also be considered a useful tool in monitoring the efficacy of specific therapies for renal and systemic diseases.

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