The Effect of Tumor Necrosis Factor-Alpha on Glomerular Epithelial Cells in Glomerular Permeability

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= **Abstract** =

Purpose : Minimal Change Disease (MCD) is the most common primary nephrotic syndrome in children. Some suggested that tumor necrosis factor-α (TNF-α) are involved in the pathogenesis of MCD.

Methods : This study was done to see the changes of plasma and urinary TNF-α, and its effect on the determination of permeability of the glomerular basement membrane (BM) contributed by heparan sulfate proteoglycan (HSPG). Study patients consisted of 19 biopsy-proven MCD children aged 2-15 years old. Both plasma and urinary TNF-α were measured. Employing the Millicell system, TNF-α was screened for the permeability factors. We examined whether TNF-α regulated BM HSPG gene expression and HS synthesis in the glomerular epithelial cells (GECs).

Results : Urinary TNF-α during relapse was significantly increased when compared with that of during remission or controls (364.4 ± 51.2 vs 155.3 ± 20.8, 36.0 ± 4.5 ng/mg · cr) ($P<0.05$). However, negative results were obtained in the permeability assay using the Millicell system. No difference was seen in the BM HSPG gene expression and HS synthesis in the GECs.

Conclusion : It seems that TNF-α may not play a disease-specific role in the pathogenesis of MCD. **(J Korean Soc Pediatr Nephrol 2004;8:1-9)**

Key Words : Glomerular epithelial cells, TNF-alpha, Minimal change nephrotic syndrome

INTRODUCTION

Minimal change disease (MCD) is the most common cause of nephrotic syndrome in children, accounting for 90 percent of cases under the age of 10 and more than 50 percent in older children [1]. It has been proposed that MCD reflects a disorder of T-lymphocytes. These T cells, which are presumably sensitive to corticosteroid and other immunosuppressants such as cyclosporin and cyclophosphamide are thought to release a cytokine that injures the glomerular epithelial cells (GECs) [2, 3]. Glomerular epithelial cell damage may lead to albuminuria in MCD by altering the metabolism of polyanion such as heparan sulfate proteoglycan (HSPG); these polyanions constitute most of the normal charge barrier to the glomerular filtration of

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macromolecules such as albumin. The albuminuria in MCD is mainly due to loss of charge-selectivity in the glomerular wall [4, 5]. The acute infusion of plasma [6-8], supernatants [6, 9-11], or fraction of supernatants [2, 12] from cultured peripheral blood mononuclear cells (PBMCs) isolated from patients with MCD into rats has been associated with an increased urinary albumin excretion and/or a decrease in number of anionic sites in the glomerular basement membrane (GBM) [2, 7-12]. The identity of permeability factor is still uncertain. Data from some investigators have suggested that tumor necrosis factor-α (TNF-α) may be active factors as determined by some experimental models of MCD or synthesis/gene expression in patients with MCD [13].

TNF-α, a cytokine that elicits a wide spectrum of inflammatory and metabolic activities, is mainly produced by monocytes and macrophages, but also by a large variety of cells [14]. It has been suggested that TNF-α could participate in the pathogenesis of glomerular damage in various models of nephritis [15-17]. Recent data suggested that patients with MCD had higher serum TNF-α levels and TNF-α production by monocytes than patients in remission and controls. TNF-α mRNA expression of PBMCs in patients with activity was increased compared to controls and patients in remission [13].

The purpose of this study was to assess the direct effect of TNF-α on GECs that are thought to be the target cell in the pathogenesis of MCD. We measured both serum and urine levels of this cytokine in remission and relapse from children with MCD. TNF-α was also tested whether they made a change in the permeability of GEC monolayers. And, GECs were directly exposed to TNF-α, then, BM HSPG gene expression and HS synthesis were determined.

MATERIALS AND METHODS

1. Patients

Nineteen children (11 boys and 8 girls) with biopsy-proven MCD, as defined by the International Study of Kidney Disease in Children (ISKDC) [1], were included in the study. Their ages ranged 2 to 15 years (average 9.5 years). Age and sex-matched 10 healthy children were also included as healthy controls. Their ages ranged 3 to 14 years (average 9.8 years). Relapse of the nephrotic syndrome was defined as massive proteinuria (>40 mg/M^2/hr in 17 patients, >2.0 urinary protein to creatinine ratio in 2 patients) and a low serum albumin level (2.5 gm/dL). We estimated the adequacy of urine collection by calculating total creatinine content in the collected urine. Because of inadequate 24 hrs urinary collection in 2 patients, they were defined as relapse by urinary protein to creatinine ratio >2.0. Serum and urine were sampled both in remission and relapse, and kept at -70℃.

2. Glomerular epithelial cells

GECs cloned from primary rat glomerular cultures were obtained from J.L. Kreisberg (San Antonio, TX, USA); the methods of cell cloning and characterization have been described previously [18].

The differential identification was based on the following criteria : (1) cobblestone appearance of the cell monolayer, (2) presence of

microvilli, (3) presence of junctional complexes suggestive of tight junctions, (4) absence of myosin, and (5) formation of 'domes' on plastic dishes when the monolayer was kept beyond confluence, suggesting unidirectional transport of sodium and water, a characteristic of polar cells such as epithelial cells. Further characterization included sensitivity to puromycin aminonucleoside, positive staining for Heymann antigen (gp330) and heparan sulfate proteoglycan core protein, and negative staining for factor VIII [19, 20]. Discussion of the visceral epithelial origin of these cells versus a parietal origin has been presented previously [20].

3. Measurement of tumor necrosis factor-α

Both plasma and urine TNF-α were determined using a TNF-α ELISA kit (Endogen, Inc., MA, USA). Fifty μL of standards or samples were added in duplicate. The plate was covered and incubated at room temperature (20-25 ℃) for 1 hr. The plate was washed three times with wash buffer provided with the kit. Fifty μL of the biotinylated antibody reagent were added to each well being utilized. The plate was covered and incubated for 1 hr at room temperature. The plate was washed three times with wash buffer. Streptavidin-HRP concentrate was diluted in dilution buffer and 100 μL of this solution was added to each well. The covered plate was incubated at room temperature for 30 mins. Then, the plate was washed three times with wash buffer. One hundred μL of premixed TMB substrate solution was added to each well. The plate was developed at room temperature in the dark for 30 mins. The reaction was stopped by adding 100 μL of the provided stop solution to each well. The absorbance of the plate was read on a plate reader at 450-550 nm. Results were calculated using graph paper or curve fitting statistical software.

4. Permeability assay using a semi-permeable membrane (the Millicell system)

GECs were grown on the surface of cellulose semi-permeable membrane (Millicell-HA, 0.45 μm culture plate insert, 12 mm diameter, Millipore Corp., MA, USA). After confluence of GECs, the medium was changed into fresh medium containing 1,000 ng/mL of TNF-α or sera from children with MCD during relapse, then, cells were incubated for 48 hrs at 37℃ in a 5% CO_2/95% air. Human serum albumin (Sigma Corp., MO, USA) were added into the basolateral compartment. And the amount of human serum albumin (HSA) filtered into apical chambers was studied using an Albumin RIA kit (Immunotech Corp., Prague, Czech Republic) by obtaining 60 μL aliquot sample from the apical side at 18 hrs after the addition of HSA. A typical experiment also included a negative control (Millicells included with medium alone) and a positive control (Millicells treated with 95% ethanol for 5 min) followed by washing and replacement with fresh medium as Pegoraro et al. [21] did.

5. Extraction of total RNA

Rat GECs were grown to confluence in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 15 mmol/L HEPES, 0.66 U/mL insulin, 100 g/mL streptomycin, and 100 U/mL penicillin at 37℃ in 5% CO_2/95% air. TNF-α (Endogen

Inc.) was added at concentrations of 0, 10, 100, and 1,000 ng/mL. Total RNA was extracted at 24 and 48 hrs after adding TNF-α by Chomczynski and Sacchi's method [22].

6. Estimation of BM HSPG mRNA expression in rat GECs

Extracted RNA was reverse-transcriptized using random primers (cDNA cycle kit, Invitrogen Corp.). BM HSPG-specific primers were synthesized from published sequences of domain-I of BM HSPG cDNA [23, 24]. The forward and reverse primers with sequences 5'-GCTGAGGGCCTACGATGG-3' and 5'-TGCCCAGGCGTGGAACT-3', respectively, were synthesized. β-actin was used as internal control, and the forward and reverse primers with sequences 5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3' and 5'-CGTCATACTCCTGCTTGCTGATCCACATC TGC-3', respectively, were synthesized. A multiplex polymerase chain reaction (PCR) using BM HSPG-specific and β-actin primers was performed.

PCR was performed in 25 cycles, each cycle consisting of denaturation at 94℃ for 1 min, annealing of primers of cDNA at 56℃ for 2 mins, and extension at 72℃ for 1.5 min. The reaction products were separated on TBE, 1.2% agarose gel. The area and size of each band on the 1.2% agarose gel was analyzed by a gel documentation system (Alphainnotech Corp., CA, USA).

7. Estimation of HS synthesis in rat GECs

GEC was labeled with 200 μCi/mL of $^{35}SO_4$ (ICN Radiochemicals, specific activity 43 mCi/mg S, carrier-free) for 12 and 24 hrs and for the last 24 hrs of 48 hrs incubation. The cell layers were dissolved in immunoprecipitation buffer containing 20 mM TRIS, pH7.5, 0.15 M NaCl, 4 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate, 1 mM PMSF, 100 mM 6-aminohexanoic acid and 5 mM benzamidine HCl. The protein content was estimated using Biorad protein assay kit (BioRad, CA, USA). Immunoprecipitation was performed employing a modified method of Ledbetter et al. [25], as previously described, using equal amounts of cell protein (50 μg) and a specific antibody against rat GBM heparan sulfate side chain (UBI, CA, USA). The radioactivity in the immunoprecipitates was measured in a beta counter.

8. Statistics

Data are presented means standard deviations (SD) unless otherwise noted. Statistical analyses were performed using the software SPSS (version 11; SPSS Inc., Chicago, IL). Statistical significance (defined as $P<0.05$) was evaluated with paired t test and non-parametric Man-Whitney U test where appropriate.

RESULTS

1. Changes of plasma and urinary TNF-α

Values of urinary TNF-α was corrected by urinary creatinine in the same urine sample. Urinary TNF-α (ng/mg · cr) were 364.4 ±51.2, 155.3±20.8 and 36.0±4.5 in relapse, remission and healthy controls, respectively. Urinary TNF-α during relapse was significantly increased compared to healthy controls and remission ($P<0.05$) (Table 1).

Plasma TNF-α (ng/mL) were 2.42±1.93,

Table 1. Changes of Plasma (p) and Urinary (u) Tumor Necrosis Factor-α (TNF-α) in Children with Minimal Change Disease During Relapse and Remission

	Relapse	Remission	Healthy controls
pTNF-α (ng/mL)	2.42±1.93 (0.63-4.01)	1.95±1.62 (0.48-3.40)	2.25±1.75 (0.40-3.81)
uTNF-α (ng/mg.cr)	364.4±51.2*, † (298.3-406.8)	155.3±20.8† (128.5-168.6)	36.0±4.5 (31.9-40.0)

*P<0.05 compared to remission, †P<0.05 compared to healthy controls

1.95±1.62 and 2.25±1.75 in relapse, remission, and healthy controls, respectively. No significant changes of plasma TNF-α were observed in children with MCD during relapse and remission (Table 1).

2. Permeability assay using the Millicell system

Sera from children with MCD caused marked albumin leakage across the GEC monolayers in the Millicell system (P<0.05). However, TNF-α did not induce greater albumin leakage than the negative control (Table 2).

Table 2. Concentrations of Human Serum Albumin Leakage with Tumor Necrosis Factor-α (TNF-α), Sera from Children with Minimal Change Disease (MCD) and Controls (unit; μg/mL)

TNF-α 1,000 ng/mL	1.0±0.3
Sera from children with MCD	27.7±2.4*
Negative control	0.6±0.2
Positive control	46.0±1.7*

*P<0.05 compared to negative controls
Negative control; incubated with medium alone
Positive control; treated with 95% ethanol for 5 min

3. Changes in abundance of BM HSPG mRNA in rat GECs

Rat GECs were incubated until confluence. Total RNA was extracted. The abundance of BM HSPG mRNA was measured at 24 hrs and at 48 hrs after adding various concentrations of TNF-α. Twenty-four hrs after adding TNF-α, the percentages of BM HSPG mRNA expression to beta-actin mRNA expression (%) were 28.3±6.7, 31.2±7.1, 25.6±4.3, and 34.3±5.5 at concentrations of 0, 10, 100, and 1,000 ng/mL of TNF-α, respectively. Forty-eight hours after adding TNF-α, they were 33.7±3.4, 29.5±5.1, 31.5±2.8, and 37.3±4.3, at concentrations of 0, 10, 100, and 1,000 ng/mL of TNF-α, respectively. TNF-α did not induce significant changes of BM HSPG mRNA expression in rats GECs (Table 3, Fig. 1).

Table 3. Changes of Heparan Sulfate Proteoglycan mRNA Abundance in Rat Glomerular Epithelial Cells at Various Concentrations of Tumor Necrosis Factor-α (TNF-α)

	24 hrs (n=5)	48 hrs (n=5)
TNF-α		
0 ng/mL	28.3±6.7	33.7±3.4
10 ng/mL	31.2±7.1	29.5±5.1
100 ng/mL	25.6±4.3	31.5±2.8
1,000 ng/mL	34.3±5.5	37.3±4.3

Unit (%)=(HSPG band density/β-actin band density)×100

4. HS synthesis in rat GECs

Whether change in BM HSPG mRNA abundance caused by TNF-α correlated with change in the synthesis of HS was examined by immunoprecipitation. There was no significant change in the synthesis of $^{35}SO_4$ labeled HS by TNF-α (Table 4).

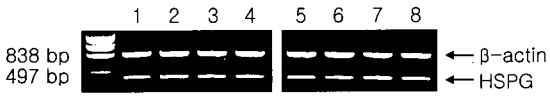

Fig. 1. No difference was observed in the abundance of heparan sulfate proteoglycan mRNA in rat glomerular epithelial cells at various concentratons of TNF-α (0, 10, 100, and 1,000 ng/mL). 1 : 24 hrs TNF-α 0 ng/mL, 2 : 24 hrs TNF-α 10 ng/mL, 3 : 24 hrs TNF-α 100 ng/mL, 4 : 24 hrs TNF-α 1,000 ng/mL, 5 : 48 hrs TNF-α 0 ng/mL, 6 : 48 hrs TNF-α 10 ng/mL, 7 : 48 hrs TNF-α 100 ng/mL, 8 : 48 hrs TNF-α 1,000 ng/mL.

Table 4. Synthesis of Heparan Sulfate in Glomerular Epithelial Cells Measured by Immunoprecipitation Using Monoclonal Antibody against Heparan Sulfate after Adding Tumor Necrosis Factor-α (TNF-α)(unit; %)

	24 hrs (n=5)	48 hrs (n=5)
TNF-α 1,000 ng/mL	101±9%	106±6%
Control	100%	100%

Control; Neither IL-8 nor TNF-α was added

DISCUSSION

The identity of vascular permeability factors which are thought to exist in the blood of patients with MCD is still uncertain. Moreover, their mechanism of action has not been exactly defined in the pathogenesis of MCD. The acute infusion of plasma, supernatants, or fraction of supernatants from cultured PBMC isolated from patients with MCD in relapse into rats has been related to an increased urinary albumin excretion with or without a decrease in the number and density of anionic sites in the GBM. Recently, researchers have suggested that hemopexin, an acute phase reactant, may be the active factor as determined by some experimental models of MCD [26, 27]. Whether this finding is applicable to patients with MCD is unclear.

Heparan sulfate proteoglycan constitutes most of the normal charge barrier to the glomerular filtration of macromolecules such as albumin, which is anionic in the physiologic pH range. The albuminuria in MCD is mainly due to loss of charge selectivity in the GBM [4, 5]. The GECs synthesize most of heparan sulfate proteoglycan in the GBM [28]. These findings suggest that GECs are target cells in the pathogenesis of MCD, in other words, vascular permeability factor (s) secreted by peripheral mononuclear cells may directly injure the GECs, consequently, altering synthesis of HS (PG) by GECs, thus resulting in albuminuria.

The Millicell system has been used to study ion fluxes across tight junction in tubular epithelial cell monolayers, and some researchers modified it to study albumin fluxes using GECs [19, 20]. Adding polycationic substances such as protamine, cationic bovine gamma globulin (BGG), or glycated proteins increased the permeability of albumin across the GEC monolayer, and the addition of heparin decreased the albumin permeability induced by cationic BGG [20]. Pegoraro et al. [21] extended the use of this Millicell technique to search for permeability factors in patients with the idiopathic nephrotic syndromes. We tested the effect of TNF-α in the Millicell system. The result was negative.

Many components are present in the GBM, the most abundant are type IV collagen chain 3, 4, 5, various laminin isoforms, and HSPGs. The main HSPGs that have been characterized until now are perlecan, agrin and recently collagen XVIII. With recently developed antibodies directed against the

HSPG core protein and the HS side chain, some researchers demonstrated a decrease in HS(PG) staining in the GBM in SLE, diabetic nephropathy, etc [29]. The number of studies dealing with HS (PG) synthesis in MCD is very limited. The studies in this field are necessary.

Bustos et al. [13] reported that patients with MCD and its variant had higher serum TNF-α levels and TNF-α production by monocytes than patients in remission and controls, and that the TNF-α mRNA levels of blood mononuclear cells in patients during relapse were increased compared to controls and to patients in remission. However, Suranyi et al.[30] described that TNF-α was significantly elevated in the plasma and urine of patients with focal segmental glomerulosclerosis and membranous glomerulonephropathy, and was normal in control subjects and patients with MCD. While Nakamura et al. [31] observed in vitro no effect on glomerular sulfate compounds, Shewring et al. [32] found that TNF-α stimulated mesangial cells to synthesize proteoglycans in a dose- and time-dependent manner. We observed that urinary TNF-α/creatinine was significantly increased in relapse. However, no significant change in the plasma TNF-α during relapse was shown. In order to validate the role of TNF-α in the pathogenesis of MCD, we measured BM HSPG gene expression and HS synthesis in the GECs after adding TNF-α. However, no significant change was seen. Accordingly, the increased urinary TNF-α level during relapse without increments of plasma TNF-α and no change in BM HSPG gene expression and HS synthesis mean that TNF-α may not play a primary role in the pathogenesis of MCD, and its elevation in the urine could be a secondary event.

In summary, TNF-α was increased in urine, not in plasma, during relapse without change in the permeability of GEC monolayers, BM HSPG gene expression and HS synthesis in the GECs. Therefore, TNF-α does not seem to play a disease-specific role in the pathogenesis of MCD; rather, its change seems to be secondary to nephrotic syndrome itself.

한 글 요 약

TNF-α가 토리 상피세포의 투과성에 미치는 영향

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목 적 : 미세변화신증후군은 소아의 원발성 신증후군의 주요 원인질환이다. 미세변화증후군의 정확한 병인기전은 아직까지 알려져 있지 않으나, 최근 TNF-α가 이 질환의 병인기전과 관련된다는 보고가 있었다. 이에 저자들은 환아의 혈청과 요에서 TNF-α의 변화를 살펴보고, TNF-α가 이 질환의 표적세포인 토리 상피세포에 미치는 직접적인 영향을 알아보고자 본 연구를 시행하였다.

방 법 : 2-15세의 미세변화신증후군 환아에게서 혈청과 요에서 TNF-α치를 측정하였고, 토리 상피세포로 도포된 Millicell system을 사용하여 TNF-α가 토리 상피세포에 의하여 형성되는 투과성에 미치는 영향을 알아보았다. 또한 TNF-α가 토리 상피세포에서 생성되어 토리 기저막의 투과성을 결정하는 물질인 heparan sulfate proteoglycan의 유전자 발현과 생성에 미치는 영향을 측정하였다.

결 과 : 미세변화신증후군의 재발시 요중 TNF-

α치 (ng/mg · cr)는 364.4±51.2로서 관해와 대조군의 155.3±20.8과 36.0±4.5에 비하여 유의하게 증가되어 있었다 ($P<0.05$). 그러나 TNF-α가 토리 상피세포의 투과성 검사와 토리 상피세포의 heparan sulfate proteoglycan의 유전자 발현과 생성에 아무런 영향을 미치지 않았다.

결 론: TNF-α는 토리 상피세포에 직접적인 영향을 미치지 않으므로 미세변화신증후군에서의 요중 TNF-α치의 증가는 질병으로 인한 이차적인 변화일 것으로 생각된다.

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