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Effect of Supernatants from Nephrotic Peripheral Blood Mononuclear Cells on ^{35}Sulfate Incorporation in Rat Glomerular Basement Membrane

EDUARDO H. GARIN AND KEVIN P. BOGGS

Department of Pediatrics, Division of Nephrology, College of Medicine, University of Florida, Gainesville, Florida 32610

ABSTRACT. In previous research, we showed that when peripheral blood mononuclear cells (PBMC) from patients with idiopathic minimal lesion nephrotic syndrome (IMLNS) in relapse were cocultured with rat glomeruli, there was an increased glomerular basement membrane (GBM) uptake of ^{35}sulfate. This study was done to determine whether the increased uptake was due to substances secreted into the nephrotic PBMC culture supernatants. ^{35}sulfate uptake in rat GBM was significantly higher when glomeruli were cocultured with PBMC from 12 IMLNS patients in relapse (geometric mean [GM] = 513 cpm/mg) than when simultaneous assays were done using either PBMC from eight control subjects (GM = 158) ($p < 0.05$) or glomeruli incubated without PBMC (GM = 275 cpm/mg) ($p < 0.01$). ^{35}sulfate uptake did not increase when glomeruli were cocultured with PBMCs from 11 MLNS patients in remission. Rat GBM ^{35}sulfate uptake was significantly higher when glomeruli were incubated in the supernatants of the PBMC cultures from 16 IMLNS patients in relapse (GM = 234 cpm/mg) than it was when glomeruli were cultured in the supernatants from normal control PBMC (GM = 126 cpm/mg; $p < 0.002$) or from glomeruli cultured alone (GM = 141 cpm/mg) ($p < 0.04$). Supernatants from PBMC cultures of 11 IMLNS patients in remission did not increase rat GBM ^{35}sulfate uptake. These data suggest that PBMC from IMLNS patients in relapse secrete a factor(s) released into supernatants that increases the ^{35}sulfate rat GBM uptake. Since sulfated compounds in the GBM play a role in glomerular permeability, this finding may have pathogenic significance. (*Pediatr Res* (19: 836–840, 1985)

Abbreviations

IMLNS, idiopathic minimal lesion nephrotic syndrome
GBM, glomerular basement membrane
PBMC, peripheral blood mononuclear cells
GM, geometric mean
cpm, counts per minute

Proteinuria in IMLNS appears to be a consequence of defective electrostatic function of the glomerular barrier to protein filtration (1). Heparan sulfate, the predominant proteoglycan in the GBM (2), is present as regularly spaced negatively charged sites along the lamina raraes of the GBM (3). This proteoglycan seems to participate in the glomerular ultrafiltration barrier, since its removal from the GBM is associated with an increase in glomerular permeability (4). Sulfate groups in heparan sulfate are an important source of anionic sites. The study of GBM sulfate metabolism may give clues to the increased glomerular permeability observed in patients with IMLNS.

Previously we reported that cocultures of PBMC from IMLNS patients in relapse and rat glomeruli showed an increased incorporation of ^{35}sulfate in the GBM of the rat glomeruli (5). This study confirmed our previous findings and demonstrated that the increased ^{35}sulfate incorporation is due to a factor secreted in the supernatants by the PBMC.

MATERIALS AND METHODS

Patients. Eighteen patients with IMLNS as defined by the International Study of Kidney Disease in Children (6) were included in the study. Fourteen were males and four were females. Their ages ranged from 2 5/12 to 21 yr with a median age of 9 yr. Eight normal adults served as controls.

Sixteen of the 18 IMLNS patients were studied during relapse. Nine of these patients were also studied during remission. The remaining two patients were studied during remission only. Patients were considered in relapse if they had massive proteinuria (3+ by Albustix or >40 mg/m^2/h) and low serum albumin (<2.5 g/dl). Patients were considered in remission when serum albumin was within normal limits and protein excretion was normal (trace or negative by Albustix or <5 mg/m^2/h).

No patient was receiving prednisone at the time of the study.

Isolation of PBMC. Peripheral blood mononuclear cells were isolated from heparinized blood by density centrifugation on a Ficoll Hypaque gradient (7). After the cells had been washed three times with Hanks' balanced salt solution, viability was assessed by trypan blue exclusion (>95% in all preparations).

Isolation of rat glomeruli. Glomeruli from Sprague-Dawley rats (100 to 350 g; Charles River, Wilmington, MA) were isolated by the method described by Fong and Drummond (8). At the end of the procedure, preparations were almost pure of tubular contaminations and consisting of virtually 100% glomeruli.

The glomeruli obtained from each rat were divided into three aliquots. One was cocultured with PBMC from nephrotic patients, another was cocultured with PBMC from normal control subjects at the same concentration as nephrotic PBMC, and the third was cultured alone. PBMC from seven of the patients in relapse and those from seven control subjects were divided into

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Reprint requests to Eduardo H Garin, M.D., Department of Pediatrics, Box J-296, JHMHC, University of Florida, College of Medicine, Gainesville, FL 32610.

two equal aliquots. One was cocultured with rat glomeruli as described above and the other was cultured alone but with ^{35}sulfate added to the culture media.

Glomeruli and PBMC were incubated in 5 ml of Basal Eagle Medium (without sulfate, Catalog no. 424-1300, Grand Island Biological Company, New York, NY). The culture medium was supplemented with 200 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 20% decomplemented fetal calf serum, and 80 μCi of ^{35}sulfate (specific activity, 209-335 mCi/mM; New England Nuclear, Boston, MA). Glomeruli and PBMC, glomeruli alone, and PBMC alone were incubated at 36° C with Petri dishes (50 × 50 mm) in a humidified atmosphere of 5% CO_2 and 10% O_2 for 48 h. At the end of incubation, the samples were centrifuged, the supernatants were collected, and the pellet was lyophilized and kept at −35° C until the GBM was isolated.

In the next experiment, glomeruli from rats were again divided into three aliquots. One was cultured in the supernatant from nephrotic PBMC and glomeruli cultures, another in the supernatant from normal PBMC and glomeruli cultures, and the third in the supernatant of rat glomeruli cultured alone. In a concomitant experiment rat glomeruli were cultured with supernatants from PBMC of patients in relapse and those of normal control subjects.

Isolation of GBM. GBM was prepared from lyophilized, isolated glomeruli by the method Meezan *et al.* (9) with minor modifications. Lyophilized samples were weighted and glomeruli were hypotonically lysed in 100 ml of 0.05% sodium azide for 12 h. Subsequently, lysed samples were treated with desoxyribonuclease (50 Kunitz units/ml) for 2 h and then digested with 4% sodium desoxycholate for 2 h. The final mixture was centrifuged, the supernatant discarded, and the pellet washed five times with distilled water by resuspension and centrifugation. The washed precipitate was dried and resuspended in Atomlight (New England Nuclear); radioactivity was counted for 1 min in a Beckman liquid scintillation counter. Results were expressed in cpm/mg dry glomerular weight. To compare ^{35}sulfate incorporation in rat GBM when glomeruli were incubated with PBMC from same patient during relapse and remission, results were expressed in cpm/mg dry glomerular weight per 10^6 PBMC.

Serum albumin was determined by the brown-creosol-green method using a Technicon Autoanalyzer II. Urinary protein was measured by the sulfosalicylic method.

Statistical analysis was performed on an IBM PC computer using NWA Statpak (Northwest Analytical Inc, Portland, OR). Programs included one-way analysis of variance, randomized, block assignment, and multiple *t* tests. Analysis of variance and differences between means were performed on the logarithms of the measured values of ^{35}sulfate uptake by the GBM.

Table 1. *^{35}Sulfate uptake by rat GBM after incubation of glomeruli with A) PBMC from IMLNS patients in relapse, B) PBMC from normal controls, and C) no PBMC*

	^{35}Sulfate uptake (cpm/mg dry glomerular wt)		
	A) Glomeruli + IMLNS PBMC	B) Glomeruli + normal PBMC	C) Glomeruli alone
	891	331	1258
	44	15	30
	1445	617	794
	135	95	145
	977	794	347
	7079	105	813
	251	562	95
	3311	16	1905
	65	87	30
	363	145	407
	794	525	562
	417	209	138
GM incorporation	513	158	275
95% prediction interval	26–10083	12–2172	18–4130

* ANOVA (F;df;*p*) 3.70; 2,22; <0.05)
Significance of differences: A *vs* B $t = 2.25$, $p < 0.05$; A *vs* C $t = 3.16$, $p < 0.01$; B *vs* C $t = 1.10$, p = NS

* ANOVA, analysis of variance; df, degree of freedom.

Table 2. *^{35}Sulfate uptake by rat GBM after incubation of glomeruli with A) PBMC from IMLNS patients in remission, B) PBMC from normal controls, and C) no PBMC*

	^{35}Sulfate uptake (cpm/mg dry glomerular wt)		
	A) Glomeruli + IMLNS PBMC	B) Glomeruli + normal PBMC	C) Glomeruli alone
	489	258	160
	496	858	863
	640	1741	875
	321	0	240
	52	190	174
	22	100	44
	76	134	156
	417	965	840
	942	658	1684
	78	504	556
	1310	1407	1020
GM incorporation	240	257	380
95% prediction interval	18–3142	4–14927	44–3317

* ANOVA (F,df,*p*) (0.82; 2,20; NS)
Significance of differences; ANOVA test p = NS

* ANOVA, analysis of variance; df, degree of freedom.

RESULTS

During relapse the serum albumin concentration of patients with IMLNS was 2.3 ± 0.3 g/dl (mean ± SD), while during remission it was 4.2 ± 0.5 g/dl. The serum creatinine concentration was 0.6 ± 0.1 mg/dl during relapse and 0.5 ± 0.1 during remission (p = NS).

^{35}Sulfate incorporation in rat GBM was significantly higher when glomeruli were cocultured with PBMC from IMLNS patients in relapse (GM = 513 cpm/mg) than in the cultures from normal control subjects (GM = 158 cpm/mg; $p < 0.05$) and in cultures of glomeruli without PBMC (GM = 275 cpm/mg; $p < 0.01$) (Table 1). Although 16 patients were studied during relapse, data on $^{35}SO_4$ uptake by GBM when glomeruli were cocultured with nephrotic PBMC were available in only 12 patients.

The ^{35}sulfate incorporation after glomeruli were cultured with PBMC from 11 IMLNS patients in remission did not differ significantly from that observed when glomeruli were incubated with PBMC from normal adults or when glomeruli were incubated alone. (ANOVA F = 2.36; degree of freedom 2,20; p = NS) (Table 2.)

Tables 3 and 4 show ^{35}sulfate incorporation in rat GBM after glomeruli were incubated in the supernatants of the previously described types of cultures. GBM ^{35}sulfate incorporation was significantly higher when glomeruli were incubated in the supernatants of PBMC from 16 IMLNS patients in relapse (GM =

234 cpm/mg) than it was when glomeruli were cultured in the supernatants from normal control PBMC (GM = 126 cpm/mg; $p < 0.002$) or from glomeruli cultured without PBMC (GM = 141 cpm/mg; $p < 0.04$) (Table 3.)

No significant differences in ^{35}sulfate GBM uptake were found when glomeruli were cultured in the supernatants from 11 PBMC of IMLNS patients in remission as compared to ^{35}sulfate incorporation when glomeruli were cultured in the supernatants from normal PBMC or from glomeruli cultured without PBMC. (ANOVA: F: 2.36, degree of freedom 2,20; p = NS) (Table 4.)

^{35}Sulfate incorporation was significantly higher ($p < 0.01$) when glomeruli were incubated in supernatants from PBMC of seven patients in relapse as compared to the incorporation observed when glomeruli were incubated in supernatants from the

Table 3. *^{35}Sulfate uptake by rat GBM after incubation of glomeruli with A) supernatants of glomeruli and PBMC from IMLNS patients in relapse cultures, B) supernatants of glomeruli and PBMC from normal controls cultures, and C) supernatant from glomeruli cultured without PBMC*

	^{35}Sulfate uptake (cpm/mg dry glomerular wt)		
	A) Glomeruli + supernatants from glomeruli + IMLNS PBMC cultures	B) Glomeruli + supernatants from glomeruli + normal PBMC cultures	C) Glomeruli + supernatants from glomeruli alone
	305	54	106
	489	310	265
	130	60	51
	220	145	171
	3363	1992	324
	430	280	408
	43	125	39
	128	35	45
	50	32	27
	98	23	337
	88	29	73
	766	658	291
	510	312	103
	616	426	390
	139	66	108
	207	150	594
GM incorporation	234	126	141
95% prediction interval	27–2042	11–1507	21–940

* ANOVA (F;df;p) (4.34; 2,30;<0.02)
Significance of differences: A *vs* B $t = 3.817$, $p < 0.002$; A *vs* C $t = 2.31$, $p < 0.04$; B *vs* C $t = 0.35$, p = NS

* ANOVA, analysis of variance; df, degree of freedom.

Table 4. *^{35}Sulfate uptake by rat GBM after incubation of glomeruli with A) supernatants from glomeruli and PBMC of IMLNS patients in remission cultures, B) supernatants from glomeruli and PBMC of normal control cultures, and C) supernatants from glomeruli cultured alone*

	^{35}Sulfate uptake (cpm/mg dry glomerular wt)		
	A) Glomeruli + supernatants from glomeruli + IMLNS cultures	B) Glomeruli + supernatants from glomeruli + normal PBMC cultures	C) Glomeruli + supernatants from glomeruli cultures alone
	554	755	98
	215	380	473
	107	274	45
	52	190	174
	254	567	97
	37	55	24
	326	13	58
	407	820	203
	200	242	120
	24	64	76
	248	54	63
GM incorporation	148	138	93
95% prediction interval	19–1127	8–2370	20–433

* ANOVA (F;df;p) (2.36; 2,20; $p = 0.12$)
Significance of differences, ANOVA test p = NS

* ANOVA, analysis of variance; df, degree of freedom.

Table 5. *^{35}Sulfate uptake by rat GBM after incubation of glomeruli with supernatants from glomeruli + PBMC and from PBMC alone**

	^{35}Sulfate uptake (cpm/mg dry glomerular wt)			
	Nephrotic		Normal control	
	Glomeruli + PBMC	PBMC	Glomeruli + PBMC	PBMC
	900	305	333	54
	4106	130	60	60
	7205	430	106	280
	685	43	534	125
	800	88	533	29
	705	98	1259	23
	421	766	211	658
GM incorporation	1202		282	89
95% prediction interval	158–9162		37–2148	9–932
Significance of differences	$p < 0.01$ $t = 3.82$		p = NS $t = 1.56$	

* PBMC were obtained from same IMLNS patients in relapse and normal controls.

Table 6. *^{35}Sulfate uptake by rat GBM after incubation of glomeruli with PBMC from IMLNS patients in remission and from same patients in relapse*

	^{35} Sulfate uptake (cpm/mg dry glomerular wt per 10^6 PBMC)	
	Glomeruli + relapse PBMC	Glomeruli + remission PBMC
	409	245
	733	238
	5542	278
	306	409
	343	268
	104	4
	400	75
	1035	21
	234	19
GM incorporation	479	83
95% prediction interval	55–4176	4–1959
Significance of difference	$p < 0.01$ $t = 3.61$	

same PBMC that had been previously cultured with glomeruli (Table 5). No significant differences in ^{35}sulfate uptake were seen between same types of supernatants but from control subjects' PBMC.

When glomeruli were incubated with PBMC from nine patients in relapse the ^{35}sulfate uptake increased significantly (GM = 479 cpm/mg dry glomerular weight per 10^6 PBMC) compared with that seen when glomeruli were incubated with PBMC from the same patients in remission. (GM = 83 cpm/mg per 10^6 PBMC) ($p < 0.01$) (Table 6.)

DISCUSSION

In this study we confirmed our initial finding of increased incorporation of ^{35}sulfate in rat GBM when glomeruli were incubated with PBMC from patients with IMLNS in relapse (5). As in our previous report, control PBMC were obtained from normal adult subjects rather than from age-, sex-matched children or adolescents. However, no significant differences in ^{35}sulfate have been found between PBMC cultures taken from patients in relapse from glomerulopathies other than IMLNS and those from normal subjects (5). This highly suggests that the PBMC-induced increased ^{35}sulfate incorporation is not age dependent. Our data as well as those of others (10) evaluating the metabolism of sulfated compounds in rat GBM *in vitro* have shown great variability. In our study, variability could have been exaggerated by the use of rats of different ages, since metabolism of proteoglycans is known to change with age (11). Moreover, rat glomeruli were incubated with different concentrations of PBMC. However, although the data are variable, the variations affected equally both experimental and control groups and thus can not explain our findings.

Our data show that the increased ^{35}sulfate uptake is due to a factor(s) present in the supernatant of the cultured PBMC. This factor is likely derived from the PBMC and not from glomeruli that had been incubated with PBMC because the effect was observed even when supernatants were obtained from PBMC cultured alone. Since density centrifugation on a Ficoll Hypaque gradient isolates lymphocytes and monocytes, either or both of these cells may release the factor in the supernatant.

When glomeruli were cultured with supernatant from glomeruli and PBMC cultures, ^{35}sulfate incorporation was less than that observed when glomeruli were cultured with PBMC. This could be due to an additional direct effect of the PBMC on glomerular metabolism. However, a more likely explanation is a decrease in the factor concentration in the supernatant, since some of it was probably adsorbed by the glomeruli in the initial culture. This is supported by the finding of an increased ^{35}sulfate uptake when glomeruli were cultured in supernatants from PBMC cultured alone as compared to supernatants from glomeruli and PBMC cultures.

Previously we reported that the finding of increased ^{35}sulfate incorporation seems to be specific for patients with IMLNS (5). Furthermore, it is not due to a heterologous reaction between human PBMC and rat glomeruli. When PBMC from normal control adults or PBMC from IMLNS patients in remission were cocultured with rat glomeruli, ^{35}sulfate uptake did not differ from that seen when glomeruli were cultured without PBMC. Moreover, it is recognized that lipoproteins (12) and prostaglandin E_2 (13), known to be present in increased concentrations in the serum of these patients (14, 15), can modulate nephrotic PBMC functions. However, this is an unlikely explanation for our finding since the PBMC were not cultured with nephrotic serum. An effect of lipoproteins and/or prostaglandins E_2 left on receptor cells after the PBMC washings is also unlikely because we did not observe an increase in ^{35}sulfate when PBMC from patients with active nephrotic syndrome and other type of glomerulopathies were cocultured with rat glomeruli (5). Finally, because the abnormal sulfate GBM metabolism was found during relapse and not during remission, this finding may have pathogenic significance.

It is currently postulated that IMLNS could result from a disorder of T lymphocyte function (16). Initial studies showing that lymphocytes from IMLNS patients produce a lymphokine (vascular permeability factor) that could increase glomerular permeability have been promising (17). However, this finding has not been confirmed (18) or has been found to be nonspecific and present in other types of nephrotic syndromes (19).

In this study, supernatants from cultures of PBMC from IMLNS in relapse did induce a change in the sulfate metabolism of rat GBM. Because the changes do not appear due to species differences, we suggest that the supernatant factor could also alter the sulfate metabolism of human GBM.

An increase in sulfate incorporation is likely associated with an increased synthesis of proteoglycan side-chains or glycoproteins in the GBM. We did not isolate the different ^{35}sulfate-labeled compounds of the rat GBM. However, studies by Brown

et al. (20) and Lemkin and Farquhar (2) have demonstrated that the ^{35}sulfate in rat GBM is incorporated into glycosaminoglycans and glycopeptides. The majority of the ^{35}sulfate is found in heparan sulfate. The glycopeptides have not been defined, but they may represent entactin, a recently described sulfated glycoprotein associated with basement membranes (21). The nephrotic PBMC may release a factor that induces increased catabolism of the GBM sulfated compounds, with subsequently increased synthesis as cells react to the increased catabolism. Conversely, the supernatant factor could directly stimulate the synthesis of proteoglycans and/or glycoproteins. Thus, the PBMC could be compensating for an already increased catabolism of sulfated compounds in the GBM. Because the sulfated compounds play a role in glomerular permeability, the answers to these questions will give us insight into the biological significance of this supernatant factor and possibly the pathogenesis of IMLNS.

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Metabolic Quotients of the Gravid Uterus of the Chronically Catheterized Guinea Pig

STEVEN M. BLOCK, JOHN W. SPARKS, ROBERT L. JOHNSON, AND FREDERICK C. BATTAGLIA

Division of Perinatal Medicine, Departments of Pediatrics, Obstetrics-Gynecology, and Physiology, University of Colorado School of Medicine, Denver, Colorado 80262

ABSTRACT. The uptake of substrates by the pregnant uterus defines the "diet" of the uterus and conceptus. In order to determine the uterine substrate uptake, catheters were placed in the femoral artery and uterine veins of guinea pigs at 40–49 days gestation. After at least 2 days recovery from perioperative stress, systemic arterial and uterine venous concentrations of oxygen, glucose, lactate, acetoacetate, β-hydroxybutyrate, acetate, and free fatty acids were measured and metabolic quotients were calculated. The glucose/O_2 quotient was 1.22 ± 0.07 (mean ± SEM), the lactate/O_2 quotient was −0.40 ± 0.09, the acetoacetate/O_2 quotient was 0.03 ± 0.02, the β-hydroxybutyrate/O_2 quotient was 0.01 ± 0.006, the acetate/O_2 quotient was 0.03 ± 0.01, and the free fatty acid/O_2 quotient was 0.24 ± 0.21. Among the substrates measured, glucose appears to be the major metabolic fuel of the pregnant guinea pig uterus, but does not account for all of the oxygen consumed by the uterus if adjusted for lactate production, since lactate is produced within the gravid uterus in large quantities. Acetoacetate and β-hydroxybutyrate are utilized in negligible amounts. (*Pediatr Res* 19: 840–845, 1985)

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Address for reprints Frederick C. Battaglia, M.D., Chairman, Department of Pediatrics, C-218, University of Colorado School of Medicine, 4200 East Ninth Avenue, Denver, CO 80262.
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