รายงานวิจัยฉบับสมบูรณ์

โครงการ ผลของสมุนไพร Ganoderma lucidum
ต่อสมดุลอิมมูนระหว่างซัยโตคายน์ที่ออกฤทธิ์ให้เกิดการอักเสบ (ทีเอช1)
กับซัยโตคายน์ที่ออกฤทธิ์ต้านการอักเสบ (ทีเอช2) กับการ
ทำงานของไตในโรคไตอักเสบเนฟโฟรสิส

โดย

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โครงการ ผลของสมุนไพร Ganoderma lucidum ต่อสมดุลอิมมูนระหว่างซัยโตคายน์ที่ออกฤทธิ์ให้เกิดการอักเสบ (ทีเอช1) กับซัยโตคายน์ที่ออกฤทธิ์ต้านการอักเสบ (ทีเอช2) กับการทำงานของไตในโรคไตอักเสบเนฟโฟรสิส

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สนับสนุนโดย ทบวงมหาวิทยาลัย และสำนักงานกองทุนสนับสนุนการวิจัย

Corrections of Immunocirculatory Imbalance and Hemodynamic Maladjustment with Ganoderma Lucidum and Vasodilators Suppress Proteinuria and Restore Renal Function Respectively, in Severe Nephrosis (FSGS)

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A progressive renal disease towards end stage renal failure and a refractory – to – be – treated proteinuria are hallmarks of FSGS nephrosis. This study investigates the role of immunocirculatory imbalance inducing glomerular endothelial dysfunction associated with hemodynamic maladjustment which is crucial to the pathogenesis of renal disease progression. In essence, serum from the patient showed proinflammatory cytokine TNF alpha activity which could enhance endothelial cell cytotoxicity and glomerular endothelial dysfunction. This is reflected by **hemodynamic maladjustment** characterized by preferential constriction of the efferent arteriole. Such constriction has a significant hemodynamic impact as follows : **Proximally**, it induces intraglomerular hypertension and capillary dilation. Detachment of podocyte from the basement membrane secondary to capillary dilation, decreases production of vascular endothelial growth factor. This would affect the survival and growth of endothelial cell. Further injury to the glomerular endothelial cell would aggravate in a viscious cycle manner, a greater magnitude of hemodynamic maladjustment and eventually a further injury to podocyte. **Distally**, it exaggeratedly reduces the peritubular capillary flow (PTCF) which supplies the tubulointerstitium. PTCF reduction and glomerular endothelial dysfunction (increased AII) activate profibrotic pathway and culminate in tubulointerstitial fibrosis. A correlation between PTCF reduction and the magnitude of TIF is observed. This forms **a new body of knowledge** that is relevant to be pathogenesis of renal disease progression.

Therepeutic corrections with (1) vasodilators (ACEI + AII receptor antagonist + calcium channel blocker + antiplatelet) is for the first time able to restore renal function (2) Ganoderma lucidum suppresses proteinuria through the correction of immunocirculatory imbalance and inhibition of endothelial cell cytotoxicity.

Publications

1. Clin Hemorheol Microcirc 2004; 31:267-272

2. Ren Fail 2004; 26:259-264

3. Clin Hemorheol Microcirc 2004, 31:197-205

4. Clin Hemorheol Microcirc 2003; 29:469-474

5. Microcirculation Annual 2005 in press

6. Ren Fail 2005 in press

สัญญาเลขที่ MRG 4680193

ชื่อโครงการ ผลของสมุนไพร Ganoderma lucidum ต่อสมดุลอิมมูนระหว่างซัยโตคายน์ที่ออกฤทธิ์ให้เกิดการอักเสบ (ทีเอช1) กับซัยโตคายน์ที่ออกฤทธิ์ต้านการอักเสบ (ทีเอช2) กับการทำงานของไตในโรคไตอักเสบเนฟโฟรสิส

ระยะเวลาโครงการ 1 กรกฎาคม 2546 – 30 มิถุนายน 2548 (2 ปี)

ชื่อหัวหน้าโครงการวิจัยผู้รับทุน รศ.พญ.ดร.นริสา ฟูตระกูล

ชื่อนักวิจัยที่ปรึกษา รศ.ดร.สุทธิลักษณ์ ปทุมราช

1. **การดำเนินงาน** ☑ ได้ดำเนินงานตามแผนที่วางไว้

2. **รายละเอียดผลการดำเนินงานของโครงการ**

2.1 **กิจกรรมที่ได้วางแผนไว้**

(1) ศึกษาในผู้ป่วยเนฟโฟรสิสชนิดรุนแรงที่มี (i) ภาวะไข่ขาวรั่วดื้อต่อการรักษาด้วยยา (ii) การรักษาด้วยยาทั่วไปไม่สามารถฟื้นฟูสมรรถภาพของไตได้

(2) การตรวจขั้นพื้นฐาน

ก. การตรวจสารซัยโตคายน์ในเลือด ได้แก่ สารซัยโตคายน์ที่ออกฤทธิ์ให้เกิดการอักเสบ (TNFα) กับสารซัยโตคายน์ที่ต้านการอักเสบ (interleukin-10)

ข. การตรวจการทำงานของไต

Creatinine clearance

Fractional excretion of magnesium

ปริมาณไข่ขาวในปัสสาวะ

การศึกษาโลหิตพลศาสตร์ของไต

ค. การศึกษา endothelial cell cytotoxicity

(3) การรักษา (i) เพื่อลดภาวะไข่ขาวรั่วด้วยยา Ganoderma lucidum ขนาด 900-1125 มก./วัน (ii) การฟื้นฟูสมรรถภาพของไตด้วยยาออกฤทธิ์ขยายหลอดเลือด ประกอบด้วย ACE inhibitor, AII receptor antagonist, calcium channel blocker และยาต้านเกร็ดเลือด

2.2 **กิจกรรมที่ได้ทำจริง**

ได้ศึกษาในผู้ป่วยเนฟโฟรสิสชนิดรุนแรงที่มีการตายของเนื้อไตร่วม (focal segmental glomerulosclerosis) และมีภาวะไข่ขาวรั่วที่ดื้อต่อการรักษา มาทำการตรวจตามแผนที่กำหนดเพื่อทราบข้อมูลพื้นฐานก่อนการรักษา ข้อมูลที่ตรวจพบมีดังนี้

(i) การศึกษาระดับซัยโตคายน์

ก่อนการรักษา ระดับสารซัยโตคายน์ TNFα ในเลือดผู้ป่วย (8.9 pg/มล) ซึ่งสูงกว่าค่าในคนปกติ (5.7 pg/มล) อัตราส่วนระหว่าง TNFα/interleukin-10 ในผู้ป่วย (3.8±1.2) ซึ่งสูงกว่าค่าในคนปกติ (2.5±0.2) ดังแสดงในรูปที่ 1 ***หลังการรักษา*** ด้วยยา Ganoderma lucidum พบว่าระดับสายซัยโตคายน์ TNFα ในเลือดผู้ป่วยลดลงเหลือ 6.9 pg/มล ระดับสารซัยโตคายน์ interleukin-10 เพิ่มขึ้นเป็น 3.2 pg/มล ในขณะเดียวกันอัตราส่วนระหว่าง TNFα/interleukin-10 ลดลงสู่ระดับปกติ

(ii) การตรวจการทำงานของไต

ก่อนการรักษา ระดับ creatinine clearance 50±24 มล/นาที/1.73ม^2 ซึ่งต่ำกว่าค่าปกติ (120 มล/นาที/ 173ม^2) ค่า fractional excretion ของ magnesium ซึ่งเป็นดัชนีวัดการทำงานของเซลล์บุท่อไตสูงผิดปกติ (6±3%) เมื่อเทียบกับค่าในคนปกติ (1.6±0.6%) แสดงว่ามีการตายของเซลล์บุท่อไตเกิดขึ้น ปริมาณไข่ขาวในปัสสาวะ (2.2±1.6 กรัม/วัน) ซึ่งสูงผิดปกติ (ค่าปกติ < 0.3 กรัม/วัน) ***หลังการรักษา*** ด้วยยาออกฤทธิ์ขยายหลอดเลือดร่วมกับ Ganoderma lucidum ค่า creatinine clearance เพิ่มขึ้นเป็น 70 มล/นาที/1.73ม^2 ค่า fractional excretion ของ magnesium ลดลงเหลือ 3.4±2% ปริมาณไข่ขาวในปัสสาวะลดลงสู่ระดับปกติ

(iii) ผลการศึกษาทางโลหิตพลศาสตร์ของไต

ก่อนการรักษา พบความผิดปกติในโลหิตพลศาสตร์ของไต คือ พบปริมาณเลือดหล่อเลี้ยงไตรวม (renal plasma flow) ลดลง 234±97 มล/นาที/1.73ม^2 (ค่าปกติ 598±41 มล/นาที/1.73ม^2) ความดันภายในหลอดเลือดโกลเมอรูลัส (PG) สูงผิดปกติ 55±2 มม ปรอท (ค่าปกติ 51±0.02 มม ปรอท) ปริมาณเลือดหล่อเลี้ยงส่วนเซลล์บุท่อไต (peritubular capillary flow) 182±81 มล/นาที/1.73ม^2 เทียบกับค่าปกติ (483±43 มล/นาที/1.73ม^2) แรงต้านทานที่ผนังหลอดเลือดนำเข้าโกลเมอรูลัส (RA) เท่ากับ 10753±6426 ดายน์.วินาที.ซม$^{-5}$ เทียบกับค่าปกติ (2440±138 ดายน์.วินาที.ซม$^{-5}$ แรงต้านทานที่ผนังหลอดเลือดออกจากโกลเมอรูลัส (RE) 12447±7082 ดายน์.วินาที.ซม$^{-5}$ ดังแสดงในตารางที่ 2 ***หลังการรักษา*** พบปริมาณ renal plasma flow เพิ่มขึ้นเป็น 399±129 มล/นาที/1.73ม^2, ความดันภายในหลอดเลือดโกลเมอรูลัสลดลงเป็นปกติ (51±0.7 มม ปรอท), ปริมาณเลือดหล่อเลี้ยงส่วนเซลล์บุท่อไตเพิ่มขึ้นเป็น 323±116 มล/นาที/1.73ม^2 แรงต้านทานที่ผนังหลอดเลือดนำเข้าโกลเมอรูลัส ลดลงเกือบปกติ (3846±1682 ดายน์.วินาที.ซม$^{-5}$) ในขณะที่แรงต้านทานที่ผนังหลอดเลือดออกจากโกลเมอรูลัสก็ลดลงเช่นกันแต่ก็ยังสูงอยู่ (6124±2670 ดายน์.วินาที.ซม$^{-5}$)

(iv) การศึกษา endothelial cell cytotoxicity

การศึกษาโดยใส่ซีรั่มของผู้ป่วยลงไปในหลอดเลี้ยงเซลล์บุผิวในหลอดเลือด พบว่าทำให้เกิดการตายของเซลล์ 28.7±11% (ค่าปกติ 1±0.6%) **เมื่อรักษาด้วยยา Ganoderma lucidum ร่วมกับยาออกฤทธิ์ขยายหลอดเลือด** พบว่าอัตราการตายของเซลล์บุผิวในหลอดเลือดลดลง เหลือ 1.9±3% ดังแสดงในตารางที่ 1

อภิปราย

การศึกษาโครงการดังกล่าวพบองค์ความรู้ใหม่ที่สำคัญเกี่ยวกับ (i) กลไกการเกิดภาวะไข่ขาวรั่วที่ดื้อต่อการรักษา และ (ii) กลไกการตายของเนื้อไตที่เกิดจากภาวะเลือดหล่อเลี้ยงไตพร่องเนื่องจากมีการหดรัดตัวผิดปกติของหลอดเลือดออกจากโกลเมอรูลัสของไต (hemodynamic maladjustment)

กลไกการเกิดภาวะไข่ขาวรั่ว พบว่าสัมพันธ์กับความผิดปกติในสมดุลภูมิคุ้มกันที่มีอัตราส่วน TNFα/interleukin-10 สูงผิดปกติ ความผิดปกติดังกล่าวเสริมให้ฤทธิ์ของ TNFα อยู่ในกระแสเลือดได้นานกว่าปกติ สาร TNFα มีฤทธิ์ทำลายเซลล์บุผิวในหลอดเลือดโดยตรง เมื่อใส่ลงไปในหลอดเพาะเลี้ยงเซลล์บุผิวในหลอดเลือดทำให้เซลล์ตาย ผลกระทบที่มีต่อเซลล์บุผิวในหลอดเลือดน่าจะเป็นสาเหตุที่ทำให้เกิดภาวะไข่ขาวรั่วได้ กลไกที่น่าจะเป็นไปได้คือ TNFα ทำลายฉนวนกั้นไข่ขาวที่ผนังหลอดเลือด ซึ่งปกติเป็นสารเคลือบผิวที่มีประจุไฟฟ้าชนิดลบเหมือนกันป้องกันมิให้ไข่ขาวเล็ดลอดออกมาในปัสสาวะ เมื่อฉนวนกั้นดังกล่าวถูกทำลายไป ไข่ขาวจะสามารถเล็ดลอดออกมา แนวคิดดังกล่าวน่าจะถูกต้อง เพราะเมื่อสมดุลภูมิคุ้มกันระหว่างอัตราส่วน TNFα/interleukin-10 ได้รับการแก้ไขให้เป็นปกติโดย Ganoderma lucidum การทดสอบพบว่าพิษในซีรั่มของผู้ป่วยหายไป โดยไม่พบว่ามีการตายของเซลล์บุผิวในหลอดเลือดเพิ่มขึ้นเมื่อใส่ซีรั่มของผู้ป่วยที่ได้รับการรักษาแล้วลงไป ในขณะเดียวกันก็พบว่าภาวะไข่ขาวรั่วได้กลับลดลงเป็นปกติ

ข้อมูลที่ได้รับจากการศึกษาทางโลหิตพลศาสตร์ นำมาซึ่งการอธิบายกลไกการตายของเนื้อไตได้อย่างสมเหตุสมผล การศึกษาทางโลหิตพลศาสตร์พบการหดรัดตัวของหลอดเลือดออกจากโกลเมอรูลัสอย่างรุนแรง (hemodynamic maladjustment) (ภาพประกอบที่ 2) การหดรัดตัวอย่างรุนแรงของหลอดเลือดดังกล่าวเกิดจากความผิดปกติในการทำงานของเซลล์บุผิวในหลอดเลือดโกลเมอรูลัสของไต ทำให้ไม่สามารถหลั่งสารออกฤทธิ์ขยายหลอดเลือดได้พอเพียง การหดรัดตัวอย่างรุนแรงของหลอดเลือดออกจากโกลเมอรูลัสของไตทำให้เกิดพยาธิสรีรที่สำคัญ 2 ประการคือ **(i) หน้าต่อจุดหดรัดตัว** เลือดไม่สามารถไหลผ่านออกไปเลี้ยงไตส่วนเซลล์บุท่อไตได้ เกิดการคั่งของเลือดในหลอดเลือดฝอยโกลเมอรูลัสของไต ทำให้ความดันภายในหลอดเลือดโกลเมอรูลัสของไตสูงขึ้น เกิดการโป่งพองของหลอดเลือดฝอยโกลเมอรูลัส การโป่งพองนั้นทำให้ตัวเซลล์บุผิวนอกของหลอดเลือด (podocyte) หลุดลอกออก podocyte ที่หลุดลอกออกมีผลทำให้การสร้างสาร vascular endothelial growth factor ที่จำเป็นต่อการเจริญเติบโตและการแบ่งตัวของเซลล์บุผิวในหลอดเลือด ซึ่งโดยปกติสร้างจากตัว podocyte ลดลง (Futrakul. Microcirculation. annual 2005 in press) การเปลี่ยนแปลงดังกล่าวจะส่งผลกระทบต่อเซลล์บุผิวในหลอดเลือด ทำให้เซลล์บุผิวในหลอดเลือดตายเพิ่มขึ้น การตายของเซลล์บุผิวในหลอดเลือดที่เพิ่มขึ้นจะส่งผลกระทบต่อเนื่อง เกิดการหดรัดตัวของหลอดเลือดออกจากโกลเมอรูลัสรุนแรงขึ้น เกิดเป็นวงจรวิกฤตที่ทำให้ podocyte หลุดลอกออกเพิ่มขึ้น **หลังต่อจุดหดรัดตัว** การหดรัดตัวทำให้ปริมาณเลือดหล่อเลี้ยงไตส่วนเซลล์บุท่อไต (peritubular capillary flow; PTCF)ลดลง. PTCF ที่ลดลงทำให้เกิดภาวะเซลล์บุท่อไตขาดเลือดอย่างต่อเนื่อง การขาดเลือดหล่อเลี้ยงไตส่วนเซลล์บุท่อไตเป็นสาเหตุสำคัญที่ทำให้เกิดการ

ตายของเนื้อไตในผู้ป่วยดังกล่าว ข้อมูลที่สนับสนุนแนวคิดดังกล่าวคือ การแก้ไขภาวะหลอดเลือดหดรัดตัวดังกล่าวด้วยยาออกฤทธิ์ขยายหลอดเลือดพบว่าทำให้ปริมาณเลือดหล่อเลี้ยงไตเพิ่มขึ้นโดยเฉพาะเลือดที่ไปหล่อเลี้ยงเซลล์บุท่อไต ปริมาณเลือดที่เพิ่มขึ้นทำให้ลดอัตราตายของเซลล์บุท่อไตลง ซึ่งสนับสนุนโดยค่าของ fractional excretion ของ magnesium สัมพันธ์โดยตรงกับอัตราตายของเนื้อไตชนิดเพิ่มพังผืด (tubulointerstitial fibrosis); Futrakul Amer J Kidney Dis 1999; 33:886-891. เพราะฉะนั้นการลดลงของค่า fractional excretion ของ magnesium หลังการรักษาเป็นดัชนีชี้บ่งทางอ้อมว่า อัตราตายของเนื้อไตลดลง หรืออีกนัยหนึ่งคือมีการแก้ไขซ่อมแซมเนื้อไตให้ดีขึ้นกว่าเดิม

การเพิ่มปริมาณเลือดหล่อเลี้ยงไตเป็นประโยชน์ต่อการซ่อมแซม การแก้ไข และสร้างเนื้อเยื่อใหม่ของไต ประโยชน์ที่เห็นชัดคือ การทำงานของไตดีขึ้นอย่างชัดเจน การฟื้นฟูสมรรถภาพของไตที่เกิดขึ้นนี้แตกต่างโดยสิ้นเชิงจากการรักษาทั่วไปที่ทำได้ดีที่สุดแค่ชลอการตายของเนื้อไตลงอย่างช้าๆเท่านั้น

โดยสรุป

งานวิจัยนี้ได้สร้างองค์ความรู้ใหม่ที่เกี่ยวกับกลไกภาวะไข่ขาวรั่วที่ดื้อต่อการรักษา และกลไกการตายของเนื้อไตที่เกิดจากการหดรัดตัวของหลอดเลือดออกจากโกลเมอรูลัสของไตที่ผิดปกติ การประยุกค์องค์ความรู้ดังกล่าวไปรักษาผู้ป่วยเนฟโฟรสิสชนิด focal segmental glomerulosclerosis สามารถฟื้นฟูสมรรถภาพการทำงานของไตให้ดีขึ้น, ป้องกันการเกิดภาวะไตวายขั้นสุดท้าย และแก้ไขภาวะไข่ขาวรั่วได้สำเร็จ เป็นการลบล้างความเชื่อที่ว่าโรคไตดังกล่าวนี้ไม่สามารถรักษาได้และมักเกิดภาวะไตวายเรื้อรังขั้นสุดท้ายในที่สุด

กิตติกรรมประกาศ

ผู้วิจัยและคณะ ขอขอบคุณการสนับสนุนงานวิจัยจากสำนักงานการอุดมศึกษาแห่งชาติ (สกอ) และสำนักงานกองทุนสนับสนุนงานวิจัย (สกว)

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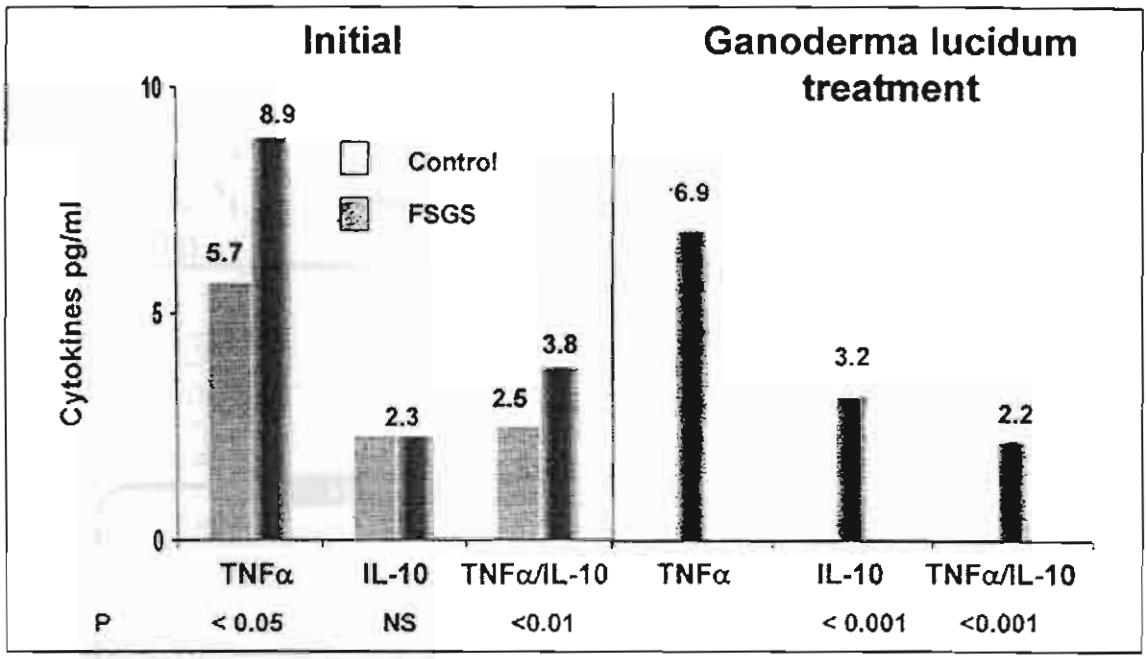


Correction of immunocirculatory balance with Ganoderma lucidum in FSGS nephrosis

ภาพประกอบที่ 1

Table 1

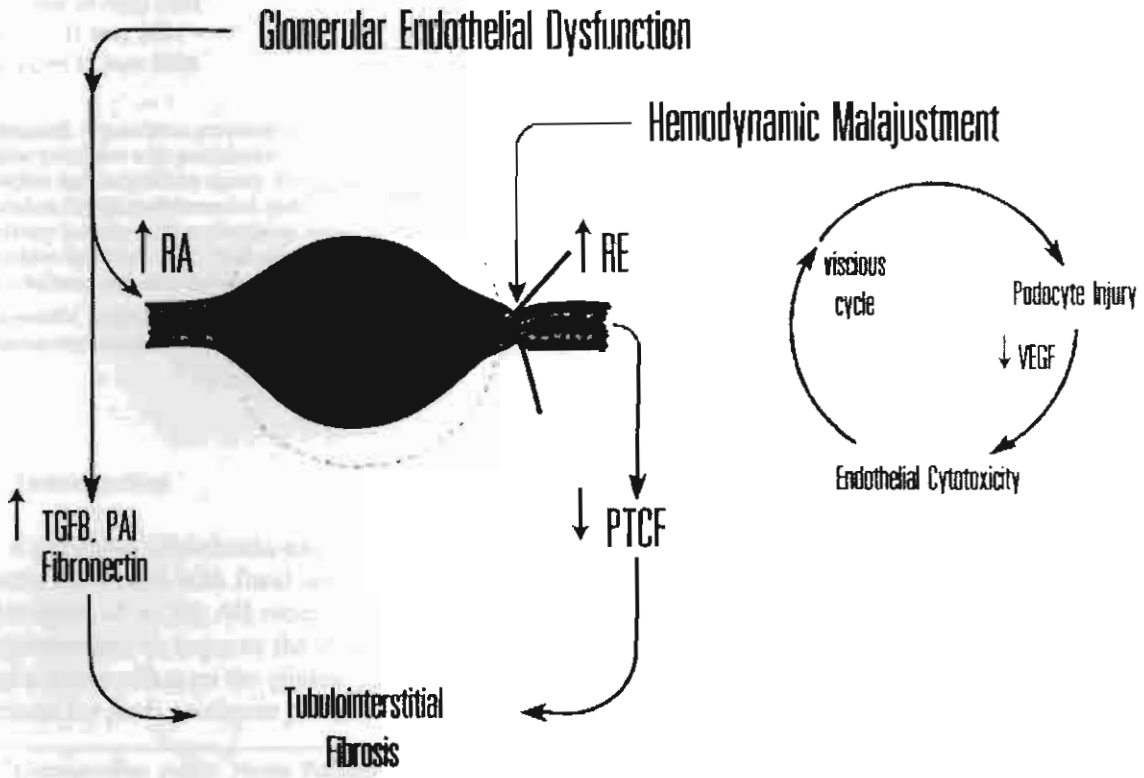
The effect of *Ganoderma lucidum* on the endothelial cell cytotoxicity and the immunocirculatory balance (cytokine studies)

	Initial	Normal	Follow-up after *Ganoderma lucidum*	*P* value
Endothelial cell cytotoxicity (%)	28.7 ± 11	1 ± 0.6	1.9 ± 3	<0.001
Immunocirculatory balance (TNF alpha/interleukin-10 ratio)	3.7 ± 1.2	2.5 ± 0.2	2.2 ± 0.4	<0.001

Table 2

Therapeutic correction of intrarenal hemodynamics in group II patients

	Normal	Before treatment	After treatment	*P*
GFR (ml/min/1.73 m^2)	114 ± 14	51 ± 23	76 ± 22	<0.01
RPF (ml/min/1.73 m^2)	598 ± 41	234 ± 97	399 ± 129	<0.001
PG (mm Hg)	51 ± 0.02	55 ± 2	51 ± 0.7	<0.001
PTCF (ml/min/1.73 m^2)	483 ± 43	182 ± 81	323 ± 116	<0.001
RA (dyne.s.cm^{-5})	2440 ± 138	10753 ± 6426	3846 ± 1682	<0.001
RE (dyne.s.cm^{-5})	3042 ± 165	12447 ± 7082	6124 ± 2670	<0.01
MAP (mm Hg)	83 ± 3	95 ± 6	79 ± 5	<0.001



ภาพประกอบที่ 2

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Ganoderma lucidum suppresses endothelial cell cytotoxicity and proteinuria in persistent proteinuric focal segmental glomerulosclerosis (FSGS) nephrosis

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Abstract. A persistent proteinuria is commonly observed in nephrotic patient with focal segmental glomerulosclerosis (FSGS) under treatment with prednisolone ± cyclophosphamide or with vasodilators (ACEI + AII receptor antagonist, calcium channel blocker and antiplatelet agent). Fourteen such patients with persistent proteinuria were subject to be treated with *Ganoderma lucidum*. Initial study revealed an enhanced endothelial cell cytotoxicity induced by patient's serum, and an altered immunocirculatory balance with predominant proinflammatory cytokine TNF alpha activity in the presence of defective anti-inflammatory cytokine interleukin-10. Treatment with *Ganoderma lucidum* suppressed endothelial cell cytotoxicity, restored immunocirculatory balance and successfully suppressed proteinuria in all of these 14 patients.

Keywords: Endothelial cell cytotoxicity, focal segmental glomerulosclerosis (FSGS), proteinuria immunocirculatory balance, *Ganoderma lucidum*

1. Introduction

A persistent proteinuria and reduction in renal perfusion are generally encountered in nephrotic patients associated with focal segmental glomerulosclerosis (FSGS). Treatment with combined formula consisting of ACEI, AII receptor antagonist, calcium channel blocker and antiplatelet agent have been demonstrated to improve the renal perfusion and restore the renal function. However, such treatment has a minor effect on the clinical persistence of proteinuria in many patients [1,2]. Although the mechanisms for such persistent proteinuria remain to be further elucidated, multiple factors, such as injury

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to podocyte, oxidative stress and hemodynamic maladjustment, have been proposed [3–7]. Recently, a glomerular endothelial dysfunction with enhanced endothelial cell cytotoxicity is documented in FSGS nephrosis [8]. Also, an immunocirculatory disturbance, associated with enhanced proinflammatory cytokine TNF alpha in the presence of defective anti-inflammatory cytokine interleukin-10, is detected in both serum and T-lymphocyte of patients with FSGS nephrosis [9,10]. The defective immunocirculatory balance is likely to induce a prolonged inflammatory effect upon glomerular endothelial function, and this may be responsible for the persistent proteinuria commonly encountered in this refractory to-be-treated group of nephrosis. The associations between enhanced endothelial cytotoxicity, altered immunocirculatory balance and persistent proteinuria documented in these FSGS patients appear to be an interesting observation. A search for therapeutic agents to suppress the proteinuria in this refractory group of patient would be desirable. In this regard, *Ganoderma lucidum* is selected to serve for this purpose because it has many pharmaceutical effects and has long been used as a home remedy. In fact, *Ganoderma lucidum*, is known to inhibit angiotensin converting enzyme activity [11] with immunomodulatory property [12]. Moreover, it is shown to protect adriamycin induced cytotoxicity in rats [13]. Since adriamycin can induce nephrotic proteinuria in experimental model in animal [5], it would be interesting to see whether *Ganoderma lucidum* can suppress proteinuria in clinical setting of human nephrosis associated with FSGS. In fact, a pilot study has recently demonstrated that *Ganoderma lucidum* can suppress proteinuria in 5 FSGS patients who have been associated with altered immunocirculatory balance and enhanced endothelial cytotoxicity [1]. The aim of this study, therefore, is to extend the observation of the effect of *Ganoderma lucidum* in other FSGS patients and also to study the mechanism of such proteinuria suppression.

2. Material and method

Fourteen patients with biopsy confirmation of FSGS with moderate degree of tubulointerstitial fibrosis who presented with persistent proteinuria under the treatment of prednisolone ± cyclophosphamide and vasodilators (ACEI + AII receptor antagonist, calcium channel blocker, antiplatelet agent) were included. The study was approved by the ethical committee of the Institution and the patients gave their informed consent. All patients had moderately impaired renal function and the degree of tubulointerstitial fibrosis correlates with the fractional excretion of magnesium [14]. With respect to the persistent proteinuria, we prospectively treated all these 14 patients with *Ganoderma lucidum*. They were subjected to the following investigations and treatment.

2.1. Endothelial cell cytotoxicity test

An endothelial cell cytotoxicity test using sera from nephrotic patients was performed as previously described [15]. In brief, the human endothelial cell line ECV 304 (American Tissue Culture Collection) in medium 199 with 10 per cent fetal bovine serum approximately 2×10^4 cells/well of 96-well tissue culture plates was incubated overnight at 37°C in a 5 per cent CO_2 atmosphere. Sera from nephrotic patients were added in duplicate wells. The culture medium and 10 per cent Triton X were used as controls that showed no cell lysis and 100 per cent lysis, respectively. The testing cultures were incubated as above for an additional 48 hours. After incubation, each well was washed with phosphate-buffered saline and then stained with crystal violet. The stained cells were lysed with acid alcohol solution, and

the optical density (OD) was determined by using a microtiter plate reader (model 3550; Biorad) at 550 nm. The percentage of cytotoxicity was calculated by using the formula as follows:

$$\% \text{ cytotoxicity} = 1 - \frac{\text{OD}_{\text{Testing}} - \text{OD}_{\text{Triton X}}}{\text{OD}_{\text{Control}} - \text{OD}_{\text{Triton X}}} \times 100.$$

2.2. *Cytokines study*

TNF alpha and interleukin-10 were measured in plasma in accordance with manufacturer's recommendations. TNF alpha and interleukin-10 were determined with commercially available ELISA kits (Predicta, provided by Genzyme Corporation, Cambridge, MA, USA). In brief, 50 μl of buffered protein base (sample deluent) was placed in each well; then 50 μl of standard or sample was added, incubated at 37°C for 90 minutes and washed with 400 μl of buffer. Thereafter, 100 μl TNF alpha biotinylated antibody was added into each test well and incubated at 37°C for 30 minutes. After washing, 100 μl of TNF alpha streptovidin reagent was added into each test well and incubated at 37°C for 15 minutes. After washing, 100 μl of working substrate solution was added into each test well and incubated for 10 minutes. The reaction was stopped with 100 μl of stop solution. The optical density was determined at 450 nm within 30 minutes after the reaction had been stopped results were expressed as pc/ml.

2.3. *Glomerular function*

Glomerular filtration rate was determined by measuring the 10-hour endogenous creatinine clearance (CCr) or glomerular filtration rate (GFR) by the radioisotope technique using ^{99m}Tc labeled diethylene triamine pentaacetic acid (DTPA) and the value was converted to the body surface area of 1.73 m^2 by the method of calculation as follows:

$$\text{Body surface area} = \frac{\text{body weight (kg)} \times 4 + 7}{90 + \text{body weight (kg)}}.$$

2.4. *Tubular function*

Indirect tubular transport was assessed by a 10-hour urinary collection as previously described [14]. Diuretic was not administered during or within 24 hours before the test. Briefly, after a regular supper, no additional food except drinking water ad lib was allowed. The patients were instructed to void at 7 PM, and then urine was collected from 7 PM to 5 AM. Clotted blood from venipuncture was drawn at the end of the test for the analysis of creatinine and magnesium levels. Urine samples were analyzed the same as blood samples by the Renal Metabolic Laboratory Unit. Analyses of (i) creatinine was determined by the method described by Faulkner and King and (ii) magnesium was determined by atomic absorption spectophotometer (model 1100 G; Perkin Elmer, Norwalk, CT). A reflection of tubular transport was derived from the determination of FE Mg which was calculated through the formula:

$$\text{FE Mg} = \frac{{}^{\text{U}}/_{\text{P}} \text{ magnesium}}{{}^{\text{U}}/_{\text{P}} \text{ creatinine}} \times 100.$$

2.5. Mode of therapy

In addition to the present medication consisting of (i) vasodilators namely angiotensin converting enzyme inhibitor (enalapril 10–40 mg/day), calcium channel blocker (isradipine 10–20 mg/day), dipyridamole 50–100 mg/day plus baby aspirin 1 gr/day, and with or without AII receptor antagonist (Losartan 50–100 mg/day). (ii) vitamin C 1000–3000 mg/day and vitamin E (400–800 IU/day), *Ganoderma lucidum* 900–1125 mg/day was additionally given to each of these 14 patients.

2.6. Statistical analysis

Values in text are expressed as mean ± SEM. The difference between pre- and post-treatment was performed by the Student's paired t-test. The difference was statistically significant when the p value was less than 0.05.

3. Results

A significant elevation of endothelial cell cytotoxicity and altered immunocirculatory balance with predominant proinflammatory cytokine TNF alpha were noted prior to the treatment (Table 1). Following treatment, endothelial cell cytotoxicity and immunocirculatory disturbance were converted back to normal. Table 2 showed altered renal function with decreased creatinine clearance, elevated FE Mg and total urinary protein. Following treatment, improvements in renal function were achieved with statistic significance.

Table 1
The effect of *Ganoderma lucidum* on the endothelial cell cytotoxicity and the immunocirculatory balance (cytokine studies)

	Initial	Normal	Follow-up after *Ganoderma lucidum*	P value
Endothelial cell cytotoxicity (%)	28.7 ± 11	1 ± 0.6	1.9 ± 3	<0.001
Immunocirculatory balance (TNF alpha/interleukin-10 ratio)	3.7 ± 1.2	2.5 ± 0.2	2.2 ± 0.4	<0.001

Table 2
The effect of *Ganoderma lucidum* on the renal function

	Initial	P value	Follow-up after *Ganoderma lucidum*	Normal
Creatinine clearance (ml/min/1.73m$^{2)}$	50 ± 24	<0.001	70 ± 23	120
Fractional excretion of magnesium (FE Mg) (%)	6 ± 3	<0.001	3.4 ± 2	1.6 ± 0.6
Total urinary protein (g/24)	2.2 ± 1.6	<0.001	0.2 ± 0.3	<0.3

4. Discussion

The result of this study indicates that *Ganoderma lucidum* can effectively suppress proteinuria in these nephrotic patients who had enhanced endothelial cell cytotoxicity and altered immunocirculatory balance. An enhanced endothelial cell cytotoxicity induced by the patient's serum in the presence of predominant activity of TNF alpha may reflect TNF alpha inducing glomerular endothelial dysfunction. A dysfunctioning glomerular endothelium favors proteinuria due to the loss of negatively charged surface (charge-selective proteinuria) [16]. In addition, a dysfunctioning glomerular endothelium also induces size-selective proteinuria secondary to the hemodynamic maladjustment which preferentially constricts the efferent arteriole. The hemodynamic maladjustment also induces podocyte detachment from the glomerular capillary basement membrane [2]. Such podocyte injury secondary to the hemodynamic mechanism as well as its direct toxic effect from the TNF alpha would exert additional mechanism inducing protienuria. The suppression of proteinuria in conjunction with the conversion of immunocirculatory balance back to normal as well as the suppression of endothelial cell cytotoxicity implies their cause – and – effect relationships even though the actual mechanism of such suppression of proteinuria remains to be determined. Nevertheless, *Ganoderma lucidum* possesses several known herbal components namely polysaccharides, triterpenoids, Ling Zhi-8 and nucleosides. The suppressive effect of proteinuria may work through their biologically active ingredients consisting of immunomodulating, anti-inflammatory, antioxidant and vasodilating effects [17–20]. Thus, the persistent proteinuria and the reduction in renal perfusion which are the common threats in nephrosis associated with FSGS can be corrected by *Ganoderma lucidum* and vasodilators respectively.

Acknowledgement

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LINICAL STUDY

Glomerular Endothelial Dysfunction in Chronic Kidney Disease

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ABSTRACT

A dysfunctioning glomerular endothelium was demonstrated in chronic kidney disease (CKD) patients by means of in vitro endothelial cell cytotoxicity test and of in vivo intrarenal hemodynamic study. An enhanced endothelial cell cytotoxicity in CKD patients was 26.5±12% as compared to 0.4±1% of control. An altered intrarenal hemodynamics revealed 1) a reduction in renal plasma flow, 190±67 mL/min/1.73 m^2 versus control 595±45 mL/min/1.73 m^2, and in peritubular capillary flow, 149±55 mL/min/1.73 m^2 versus control 479±46 mL/min/1.73 m^2, 2) an elevated intraglomerular hydrostatic pressure, 55±2 mmHg versus control 51 mmHg, elevated afferent arteriolar resistance, 13184 dyne.s.cm^{-5} versus control 2443±154 dyne.s.cm^{-5}, and elevated efferent arteriolar resistance, 13591±7591 dyne.s.cm^{-5} versus control 3062±177 dyne.s.cm^{-5}. Both enhanced endothelial cell cytotoxicity and altered intrarenal hemodynamics reflect glomerular endothelial dysfunction which is likely responsible for the renal disease progression in CKD.

Key Words: Glomerular endothelial dysfunction; Hemodynamics; Endothelial cell cytotoxicity.

INTRODUCTION

By virtue of its location at the interface between the underlying glomerular structures and blood flow-inducing mechanical shear stress, as well as containing blood elements and a variety of mediators such as inflammatory factors, metabolic products (reactive oxygen species), hemostatic triggers and vasoactive peptides, the glomerular endothelial or postglomerular capillary endothelial cell is likely to be affected by

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mechanical, immunologic and hemodynamic stress and thereby maintain finely tuned homeostasis under normal condition and under pathological conditions is disturbed with altered homeostasis.[1-3] The glomerular endothelial cell under normal function expresses vasodilators, namely prostacyclin, nitric oxide and anticoagulant surface consisting of negatively charged surface and thereby allows a free flowing of noncoagulant blood. In essence, the normal renal plasma flow (RPF) is approximately 600 mL/min/1.73 m^2. One hundred and twenty mL/min/1.73 m^2 of RPF is filtrated as glomerular filtration rate and the remaining plasma (480 mL/min/1.73 m^2) is allowed to pass through the efferent arteriole as peritubular capillary flow, which supplies the tubulointerstitial structure.

In the diseased state, most of the injurious or toxic substances gain access to the glomerular endothelial cell via the circulatory pathway. Any injury to the glomerular structure is likely to affect the glomerular endothelial cell. In this regard, a glomerular endothelial dysfunction has been substantiated in a variety of glomerulonephropathies. A dysfunctioning glomerular endothelium would express 1) a loss of negatively charged surface which is reflected by the charge-selective-type proteinuria[4,5] as well as by the altered hemostasis, namely increased consumption of platelet and fibrinogen (shortened platelet half life and fibrinogen half life, respectively) such as that encountered in acute poststreptococcal glomerulonephritis, lupus nephritis, nephrosis associated with focal segmental glomerulosclerosis and mesangial proliferation.[6-9] 2) a provasoconstrictive state due to the defective release of vasodilator in conjunction with the enhanced release of vasoconstrictors such as angiotensin II, endothelin, thromboxane A_2 etc., of which it is reflected by altered intrarenal hemodynamics. Indeed, an abnormal intrarenal hemodynamics has been consistently observed in a variety of glomerulonephropathies which is characterized by a reduction in RPF and peritubular capillary flow, and an elevated intraglomerular hydrostatic pressure and efferent arteriolar resistance.[10-13] In addition, we also observed a progressive reduction in renal perfusion as the disease severity progresses. Inasmuch as metabolic factor such as reactive oxygen species, lipid, glycation end product, cytokines have been found to be elevated in chronic kidney patients, any of these would have impact on the endothelial cell.[14-19] It is, therefore the purpose of this study to determine whether there is any evidence of glomerular endothelial dysfunction which is determined by in vitro endothelial cell cytotoxicity test and by in vivo intrarenal hemodynamic study.

MATERIALS AND METHODS

Seventeen patients associated with chronic kidney diseases and impaired renal function (mean serum creatinine 3±1 mg/dL) was subject to the following studies.

Endothelial Cell Cytotoxicity

An endothelial cell cytotoxicity induced by patients' serum was performed as previously described.[20] In brief, the human endothelial cell line ECV 304 (American tissue culture collection) in medium 199 with 10% fetal bovine serum, approximately 2×10^4 cells/well of 96-well tissue culture plates was incubated overnight at 37°C in a 5% CO_2 atmosphere. Sera from patients with chronic kidney disease were added in duplicate well. The culture medium and 10% Triton X were used as control that showed no cell lysis and 100% cell lysis, respectively. The testing cultures were incubated as above for an additional 48 h. After incubation, each well was washed with phosphate-buffered saline and then stained with crystal violet. The stained cells were lysed with acid–alcohol solution, and the optical density (OD) was determined by using a microtiter plate reader (model 3550; Biorad) at 550 nm. The percentage of cytotoxicity was calculated by

$$\text{Percent cytotoxicity} = 1-\frac{OD_{testing}-OD_{Triton\ X}}{OD_{Control}-OD_{Triton\ X}}\times100$$

Intrarenal Hemodynamics

Simultaneous assessment of effective renal plasma flow (RPF) using ^{131}I-labeled orthoiodohippuric acid (hippuran) and of glomerular filtration rate (GFR) using ^{99m}Tc-labeled DTPA were determined.[21] Intrarenal hemodynamics were calculated and based on modified Gomez's equation. Clinical data such as mean arterial pressure (MAP) was strictly recorded.

For calculation purpose, the effective filtration pressure across the glomerular capillaries (PF) is assumed to be 35 mmHg when the blood pressure is normal (BP 120/80 mmHg or less) and 40 mmHg when the blood pressure is high (BP>120/80 mmHg).[10] The hydrostatic pressure in Bowman's space (Ht) is assumed to be 10 mmHg, the renal plasma flow and

GFR are in mL/sec/1.73 m^2. From the above assumptions, the following equations are derived

$$GFR = KFG \times PF \quad (1)$$

where KFG, PF are the gross filtration coefficient of glomerular capillaries (mL/sec/mmHg) and effective pressure across the glomerular capillaries respectively

$$PG = PF + Ht + 5\left(\frac{TP}{FF} \times \log e \frac{1}{1 - FF} - 2\right) \quad (2)$$

where PG, Ht, FF are glomeruiar hydrostatic pressure, hydrostatic pressure in Bowman's space, plasma total protein (gram) and filtration fraction respectively

$$RA = \frac{MAP - PG}{RBF} \times 1328 \quad (3)$$

where RA, MAP, RBF are afferent arteriolar resistance in dyne.s.cm^{-5}, mean arterial pressure=diastolic pressure+$^1/_3$ pulse pressure and renal blood flow $\frac{RPF \times 100}{100 - \text{hematocrit}}$ respectively

$$RE = \frac{PF \times 1328}{RBF - GFR}$$

where RE is efferent arteriolar resistance in dyne.s.cm^{-5}.

STATISTICAL ANALYSIS

Values in text are expressed as mean±SEM. Nonparametric ANOVA was used to establish the significance of between group differences. The difference was statistically significant when the P value was less than 0.05.

RESULTS

1. The value of endothelial cell cytotoxicity in these CKD patients was 26.5±12% versus control 0.4±1% (Fig. 1).
2. The values of intrarenal hemodynamic study observed in CKD patients versus controls were as follows: men arterial pressure 113±17 mmHg versus control 83±3 mmHg, $p<.01$; glomerular filtration rate 41±20 mL/min/1.73 m^2 versus control 116±16 mL/min/1.73 m^2, $p<.001$; renal plasma flow 190±67 mL/min/1.73 m^2 versus control 595±45 mL/min/1.73 m^2, $p<.001$; filtration fraction 0.22±0.09 versus control 0.19±0.02, p NS; peritubular capillary flow 149±55 mL/min/1.73 m^2 versus control 479±46 mL/min/1.73 m^2, $p<.001$; intraglomerular hydrostatic pressure 55±2 mmHg versus control 51±0 mmHg, $p<.05$; afferent arteriolar resistance 13184± 7540 dyne.s.cm^{-5} versus control 2443± 154 dyne.s.cm^{-5}, $p<.01$; efferent arteriolar resistance 13591±7591 dyne.s.cm^{-5} versus control 3062±177 dyne.s.cm^{-5}, $p<.01$.

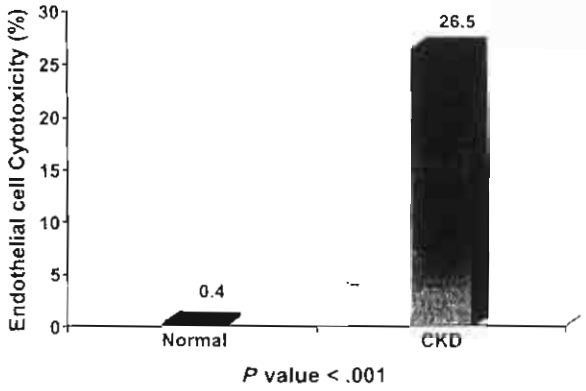

Figure 1. Enhanced endothelial cell cytotoxicity in chronic kidney disease. (*View this art in color at www.dekker.com.*)

DISCUSSION

In this study, a glomerular endothelial dysfunction is determined by an in vitro endothelial cell cytotoxicity test as well as by an in vivo intrarenal hemodynamic study. The in vitro endothelial cell cytotoxicity indicates that the patient's serum is capable of enhancing endothelial cell cytotoxicity. This implies that certain toxic substances in the serum are responsible for such phenomenon. In this regard, the glomerular endothelial dysfunction in vivo determined by the intrarenal hemodynamic study also supports the in vitro endothelial cytotoxicity test. A significant reduction in RPF and PTCF and a profound elevation in afferent and efferent arteriolar resistances have been consistently observed. These findings indicate that the glomerular endothelial function is defective in releasing the endothelium dependent vasodilators and/or enhancing the release of vasoconstrictors.

The preceding information concerning the altered intrarenal hemodynamics as well as the enhanced endothelial cytotoxicity may be relevant to the pathogenesis of renal disease progression. An enhanced endothelial cell cytotoxicity if allowed to be persistent,

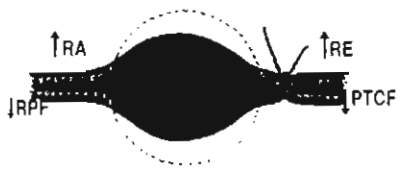

Figure 2. Intrarenal hemodynamic alterations in chronic kidney disease. *(View this art in color at www.dekker.com.)*

can induce a sustained injury to the glomerular endothelial structure and function, and such pathological state may be responsible for the progressive reduction in renal perfusion as the disease severity progresses or for the profound reduction in renal perfusion observed in these chronic kidney patients. Under this pathological state, the postglomerular or peritubular capillary endothelium is also affected. Bohle[22] and Yenrudi[23] denote an inverse correlation between the widening of the interstitium and the peritubular capillary patency or flow. Recently, a defective factor VIII staining of peritubular capillary endothelium has been demonstrated to be correlated with the magnitude of tubulointerstitial fibrosis in chronic kidney disease.[24]

An injury to the glomerular and peritubular capillary endothelium would induce a hemodynamic maladjustment by preferential constriction at the efferent arteriole. Such a constriction exerts 2 significant hemodynamic impacts. Proximal to the efferent arteriolar constriction, it elevates intraglomerular hydrostatic pressure (PG) by which it induces distension of the glomerular capillary loop. This would detach the podocyte from the basement membrane. An injury to he podocyte would decrease the production of vascular endothelial growth factor which is essential to the regeneration and survival of endothelial cells.[25] Thus, it has been a general consensus that podocyte injury is the pathological hallmark of kidney pathology observed in severe forms of kidney disease.[26–29] Distal to the efferent arteriolar constriction, it exaggeratedly reduces the PTCF which supplies the tubulointerstitium (Fig. 2). A sustained reduction in PTCF would not only induce ischemic injury but also aggravate the profibrogenic pathway and eventually culminate in the development of tubulointerstitial fibrosis.[16,17,30,31] The cause-and-effect relationship between renal perfusion and the tubulointerstitial fibrosis has recently been substantiated by the fact that renal perfusion deficit precedes the development of tubulointerstitial fibrosis.[32]

The preceding conceptual view of glomerular endothelial dysfunction which determines the renal perfusion and the mechanism of renal disease progression has been further supported by the therapeutic strategy aiming to improve the glomerular endothelial function and to correct the hemodynamic maladjustment. A restoration of glomerular endothelial function and correction of hemodynamic maladjustment can be accomplished by multidrugs consisting of antioxidant, lipid-lowering substance, vasodilators, namely angiotensin converting enzyme inhibitor, angiotensin II receptor antagonist, calcium channel blocker; and drugs that have impact on hemorheologic improvement such as antiplatelet agent.[33–35] It has been consistently observed that renal function is usually improved and well maintained following the improvement in renal hemodynamics.

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S Press

Correction of peritubular capillary flow reduction with vasodilators restores function in focal segmental glomerulosclerotic nephrosis

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stract. Due to the previously therapeutic failure in treating eleven focal segmental glomerulosis (FSGS) nephrotic patients oup I) with prednisolone, cyclophosphamide and antihypertensive agents (reserpine, hydralazine or prazosin) who all entered l-stage renal disease, we prospectively evaluate 18 FSGS nephrotic patients who have been treated with combined formula sisting of ACEI, AIIRA, CCB, antiplatelet ± heparin; group II. All the patients were subject to renal function studies ely creatinine clearance, fractional excretion of magnesium (FE Mg) and intrarenal hemodynamics. Treatment outcome atients in group II was comparatively assessed before and after therapy. Clinical profiles were comparatively matched ween groups I and II. The intrarenal hemodynamic study in all nephrotic patients revealed hemodynamic maladjustment racterized by preferential constriction at the efferent arteriole. Such constriction induced intraglomerular hypertension and geratedly reduced peritubular capillary flow (PTCF). Following treatment with combined formula (group II), reductions in rent arteriolar resistance and intraglomerular hydrostatic pressure were observed in conjunction with the increases in PTCF glomerular filtration rate in all 18 patients. Correction of hemodynamic maladjustment with combined formula effectively ores renal function and thereby prevents the renal disease progression in FSGS nephrosis.

words: Hemodynamic maladjustment, peritubular capillary flow, renal arteriolar resistance, FE Mg, vasodilators

Introduction

lthough treatment of nephrotic syndrome associated with focal segmental glomerulosclerosis GS) can be successfully accomplished with high dose steroid therapy [1] in the early course of ase when most renal function is well preserved, the fate of therapeutic outcome in the later course isease when renal function has already been impaired, is rather unfavourable and generally associ- with disease progression destined for end stage renal failure [2–4]. Therapeutic failure to the con- ional treatment requires an alternative therapeutic strategy to circumvent the disease progression. re are empirical evidence to support the role of hemodynamically mediated mechanism of renal

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disease progression in this severe renal disease. Bohle and associates [5] noted an inverse correlation between postglomerular capillary patency and the development of tubulointerstitial fibrosis in idiopathic nephrotic syndrome. Yenrudi and associates [6] also demonstrated an inverse correlation between renal perfusion and a relative area of renal cortical interstitium in nephrosis. Futrakul [7] demonstrated an endothelial factor VIII defect in renal microcirculation correlating with the severity of nephrosis. Loss of glomerular endothelium was substantiated to correlate directly with the development of glomerulosclerosis and renal disease progression [8–11]. Furthermore, chronic ischemia [12] and chronic hypoxia [13] were delineated in a variety of severe glomerulonephropathies associated with disease progression.

In accordance with the preceding view, we would like to present the intrarenal hemodynamic abnormality observed in these nephrotic patients with FSGS, which would be relevant to the pathogenesis of renal disease progression and also the effective prevention of renal disease progression by correcting the hemodynamic maladjustment with combined formula.

2. Material and method

There were 2 groups of nephrotic patients with FSGS. Prior to 1995, eleven patients (group I) were treated with prednisolone, cyclophosphamide, antihypertensive agents (reserpine, hydralazine or prazosin) and all entered end-stage renal disease. Since then we have prospectively studied a new therapeutic trial with combined formula (angiotension converting enzyme inhibitor (ACEI), AII receptor antagonist (AIIRA), calcium channel blocker (CCB), antiplatelet (dipyridamole) ± heparin) in 20 nephrotic patients with FSGS (group II). The study was approved by the ethical committee of the Institution and the patients gave their informed consent. The inclusion criteria were as follows: moderately impaired renal function, had intrarenal hemodynamic study and renal functions such as creatinine clearance, FE Na and urinary protein, had been followed up for a minimal period of 18 months. patients with the following conditions during the study were excluded: poor compliance or loss of followup. Of these 20 nephrotic patients, 2 patients were excluded from the study due to poor compliance. The remaining 18 patients had completed the study.

2.1. *Intrarenal hemodynamic study (vascular function)*

Simultaneous assessments of effective renal plasma flow (RPF) using ^{131}I-labelled orthoiodohippuric acid (hippuran) and of glomerular filtration rate (GFR) using ^{99m}Tc-labelled DTPA were determined in 31 nephrotic patients and in 5 normal controls by the previously described method [14]. Intrarenal hemodynamics were calculated and based on modified Gomez's equation [15]. For calculation purpose, the effective filtration pressure across the glomerular capillary (PF) is assumed to be 35 mm Hg where the blood pressure is normal (BP 120/80 mm Hg or less) and 40 mm Hg when the blood pressure is high (BP > 120/80 mm Hg) [16]. The hydrostatic pressure in Bowman's space (Ht) is assumed to be 10 mm Hg [15]; the RPF and GFR are in ml/s/1.73 m^2. From the above assumptions, three equations are derived as follows:

$$PG = PF + Ht + 5\left(\frac{TP}{FF} \times \log_e \frac{1}{1 - FF} - 2\right), \quad (1)$$

ere PG, Ht, TP, FF are glomerular hydrostatic pressure, pressure in Bowman's space, plasma total ein (g/100 ml) and filtration fraction, respectively.

$$RA = \frac{MAP - PG}{RBF} \times 1328, \quad (2)$$

ere RA, MAP, RBF are afferent arteriolar resistance in dyne.s/cm^5, mean arterial pressure (= diastolic ssure + 1/3 pulse pressure) and renal blood flow = RPF × 100/(100 − hematocrit), respectively.

$$RE = \frac{PF}{RBF - GFR} \times 1328, \quad (3)$$

ere RE is efferent arteriolar resistance in dyne.s/cm^5.

The peritubular capillary flow (PTCF) is derived from the substraction of GFR from RPF and is in l/min/1.73 m^2.

2. Glomerular function

Glomerular function was performed by measuring the 10-h endogenous creatinine clearance (CCr) glomerular filtration rate (GFR) by the radioisotope technique using ^{99m}Tc-labeled diethylene triamine taaccetic acid (DTPA) and the value was converted to the body surface area of 1.73m^2 by the method calculation:

$$\text{Body surface area} = \frac{\text{body weight (kg)} \times 4 + 7}{90 + \text{body weight (kg)}}.$$

3. Tubular function

Indirect tubular transport was assessed by fractional excretion of magnesium (FE Mg) through a 10-ur urinary collection as previously described [17]. No diuretic was administered during or within hours before the test. Briefly, after a regular supper, no additional food except drinking water ad lib s allowed. The patients were instructed to void at 7 PM, and the urine was collected from 7 PM to AM. Clotted blood from venipuncture was drawn at the end of the test for the analysis of creatinine d magnesium levels. Urine samples were analyzed the same as blood samples by the Renal Metabolic oratory Unit. Analysis of (i) creatinine was determined by the method described by Faulkner and ng and (ii) magnesium was determined by Atomic Absorption Spectrophotometer (model 1100 G; kin Elmer, Norwalk, CT). A reflection of tubular transport was derived from the determination of Mg which was calculated through the formula:

$$\text{FE Mg} = \frac{\text{urine magnesium}}{\text{plasma magnesium}} \times \frac{\text{plasma creatinine}}{\text{urine creatinine}} \times 100.$$

e normal value of FE Mg is $\leqslant$2.2 per cent [5].

4. Mode of therapy

All 11 patients in group I were treated with prednisolone 1–2 mg/kg/day, cyclophosphamide 1 mg/kg/day and antihypertensive agents such as reserpine, hydralazine or prazosin. Eighteen patients in group II were treated with enhanced renal perfusion formula consisting of angiotensin converting enzyme inhibitor (enalapril 10–40 mg/day), AII receptor antagonist (Losartan 50–100 mg/day), calcium channel blocker (isradipine 10–20 mg/day), dipyridamole 100 mg/day plus baby aspirin gr 1/day and subcutaneous heparin

5. Renal histopathologic study

The morphometric analysis was performed on the renal biopsied specimens by the method described elsewhere [18]. In brief, the kidney tissue was fixed in 4% buffered formalin and embedded in paraffin. Sections (2 μm) were prepared and stained with haematoxylin–eosin, periodic acid Schiff reagent (PAS), silver methenamine and Massons Trichrome. At least eight serial sections were prepared from each case and examined. The number of glomeruli varied from 10 to 35. The tubulointerstitial fibrosis (TIF) was quantitated in a single blind fashion by a pathologist who had no information regarding the hemodynamic value of each individual. The widening of the interstitium was assessed by a point-count technique using a counting grid. This manner would cover most area in the renal cortex which was ascertained by determining at $\times 100$ magnification and the result obtained was expressed as per cent.

6. Statistical analysis

Comparison of the sample mean of two quantitative variables was determined by the non-parametric method using the Mann–Whitney test. The difference between groups was performed by Student's unpaired t-test. The difference between before and after treatment values was performed by Student's paired t test. The linear regression analysis was used to correlate two quantitative variables. The scatter plot was the first step to correlate between two continuous variables. The Pearson correlation coefficient (r) was used to quantify the strength of the linear relationship. The method of least square was calculated to estimate the regression equation ($y = a \pm bx$) if the scatter plot seemed to be linear. Some relationship of the scatter plot data which had shown to be curvilinear, was further analysed to meet the criteria of straight line. P values below 0.05 were considered to be significant.

3. Results

Table 1 showed mean values of age, mean arterial pressure, creatinine clearance, FE Mg and urine protein of patients in group I which did not different statistically from patients in group II.

7. Intrarenal hemodynamics

The mean initial GFR, RPF and PTCF were significantly decreased. The mean initial PG, RA and RE were significantly elevated. Following treatment in 18 patients of group II with enhanced perfusion formula, improvements of intrarenal hemodynamics and renal function were observed (Table 2). GFR, RPF and PTCF were significantly increased. PG returned to normal, RA and RE were markedly decreased. CCr was significantly increased from 50 ± 20 to 70 ± 16 ml/min/1.73 m^2; $p < 0.001$. Tubular function

Table 1
Clinical profile in groups I and II of FSGS nephrosis

	Group I ($n = 11$)	Group II ($n = 18$)	P
Age	19 ± 2	20 ± 3	NS
Sex (male/female)	7/4	12/6	NS
MAP (mm Hg)	97 ± 10	95 ± 6	NS
CCr (ml/min/1.73 m^2)	47 ± 25	50 ± 19	NS
FE Mg (%)	6.3 ± 2	6 ± 2	NS
Urine protein (g/24 h)	3.2 ± 0.7	3 ± 0.8	NS
Duration of follow-up (mos.)	77 ± 24	97 ± 33	NS

Table 2
Therapeutic correction of intrarenal hemodynamics in group II patients

	Normal	Before treatment	After treatment	P
GFR (ml/min/1.73 m^2)	114 ± 14	51 ± 23	76 ± 22	<0.01
RPF (ml/min/1.73 m^2)	598 ± 41	234 ± 97	399 ± 129	<0.001
PG (mm Hg)	51 ± 0.02	55 ± 2	51 ± 0.7	<0.001
PTCF (ml/min/1.73 m^2)	483 ± 43	182 ± 81	323 ± 116	<0.001
RA (dyne.s.cm^{-5})	2440 ± 138	10753 ± 6426	3846 ± 1682	<0.001
RE (dyne.s.cm^{-5})	3042 ± 165	12447 ± 7082	6124 ± 2670	<0.01
MAP (mm Hg)	83 ± 3	95 ± 6	79 ± 5	<0.001

Table 3
Hemodynamic progression in group I patient under conventional therapy

	Before treatment	After treatment
GFR (ml/min/1.73 m^2)	60 ± 21	27 ± 15
RPF (ml/min/1.73 m^2)	218 ± 80	141 ± 70
PG (mm Hg)	56 ± 3	56 ± 0.8
PTCF (ml/min/1.73 m^2)	158 ± 63	114 ± 56
RA (dyne.s.cm^{-5})	10538 ± 2892	28195 ± 11987
RE (dyne.s.cm^{-5})	12147 ± 5217	22815 ± 9534
MAP (mm Hg)	97 ± 10	110 ± 7

was also improved following treatment with a decrease of FE Mg from 6 ± 2 to 3.2 ± 1.7 per cent; $p < 0.001$. The total urinary protein decreased significantly from 3 ± 0.8 to 0.6 ± 0.3 g/24 h; $p < 0.001$. All 18 patients in group II have survived after 97 ± 33 months of follow-up. In contrast, all of 11 patients under conventional therapy showed progressive reduction in PTCF, markedly and predominantly elevated afferent arteriolar resistance, and all patients entered end stage renal failure (Table 3).

2. *Histopathology observation*

The renal histopathology observed in these nephrotic patients associated with FSGS revealed a widening of the tubulointerstitium with generalized tubulointerstitial fibrosis. The mean value of quantitative score of tubulointerstitial fibrosis was 58 ± 14 percent.

4. Discussion

Both groups of patients in this study were initially presented with similar degree of clinical sev and functional impairment (Table 1). Patients with such degree of renal functional impairment ust resist to the conventional therapy with prednisolone and immunosuppressant and follow a progres course destined for end-stage renal disease [1–4]. The result in this study also supports such view s all eleven patients in group I entered end stage renal failure. In contrast to the therapeutic failure conventional therapy in group I, clinical improvement in response to the combined formula has b substantiated in all 18 patients in group II.

The explanation for such therapeutic benefit can be substantiated through the correction of the served intrarenal hemodynamic alteration. All of these patients associated with FSGS showed intrar hemodynamic maladjustment characterized by a preferential constriction at the efferent arteriole. S a constriction exerts 2 significant hemodynamic impacts which is relevant to the pathogenesis of r disease progression. Proximal to the constriction, it induces intraglomerular hypertension and disten of the glomerular capillary loop. Kriz [19] and Rennke [20] had elegantly observed that the distensio the glomerular capillary loop causes detachment of the podocyte from the basement membrane due t non-proliferative and non-distensible characteristics [21]. Injury to the podocyte has been delineated 23] and such injury reduces the production of vascular endothelial growth factor which is essential to proliferation and regeneration of endothelial cell [24]. Further injury to the glomerular endothelial would aggravate in a viscious cycle manner, the hemodynamic maladjustment and the podocyte inj Distal to the efferent arteriolar constriction, it exaggeratedly reduces the peritubular capillary flow wl supplies the tubulointerstitial structure. Peritubular capillary injury has been substantially documente severe glomerulonephritides associated with tubulointerstitial fibrosis. Increased destruction of the p glomerular capillary which is reflected by the absence of capillary patency [5], reduced endothelial fa VIII staining in the renal microcirculation [6] and reduced peritubular capillary flow [7], correlates w the magnitude of tubulointerstitial fibrosis and renal disease progression [8,25]. Such finding supp the concepts of chronic ischemia [12] and chronic hypoxia [13,26] which also activate the profibr pathway and culminate in the development of tubulointerstitial fibrosis [27]. Peritubular capillary fl reduction has been substantiated in a variety of severe glomerulonephritides namely mesangial prolife tive nephrosis with tubulointerstitial fibrosis, membranoproliferative glomerulonephritis, IgA nephro thy, reflux nephropathy, diabetic nephropathy and chronic kidney diseases [28–31]. It has recently b observed that there is an inverse correlation between peritubular capillary flow reduction and the m nitude of tubulointerstitial fibrosis (Fig. 1) and that the reduction in peritubular capillary flow prece the development of tubulointerstitial fibrosis [28]. The preceding information implies that the reduct in peritubular capillary flow determines the renal disease progression. The progressive deterioration intrarenal hemodynamics observed in group I patients entering end stage renal failure is an interest observation. The shifting pattern from the predominant efferent arteriolar constriction observed in early course of FSGS nephrosis to the preferential afferent arteriolar constriction documented in the l course needs explanation. It is noted that the continuous defect of tubular reabsorption allows access unabsorbed solutes from the proximal tubule to reach macula densa where such excessive solute l would increase intraluminal osmolarity and trigger the juxtaglomerular apparatus mechanism that duces afferent arteriolar constriction. In addition, a continuous elevation of intraglomerular hydrost pressure secondary to the efferent arteriolar constriction in accordance with the La Place's law [3 would translate into an increase in biaxial tensile stress which in turn transmits into the wall of the ferent arteriole and subsequently induces proliferation of the vascular smooth muscle cell in order

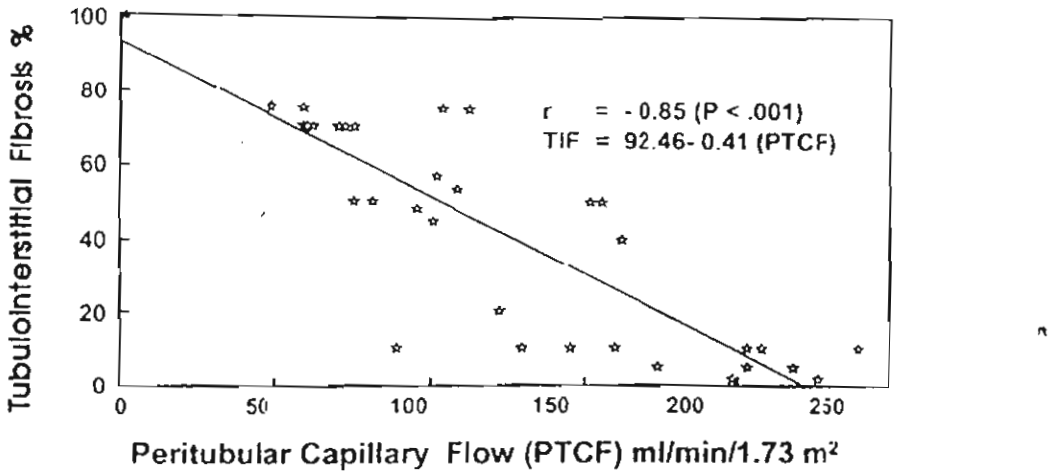

Fig. 1. Correlation between peritubular capillary flow (PTCF) and the magnitude of tubulointerstitial fibrosis.

tralize the increased biaxial tensile stress. This is a kind of trade-off hypothesis which eventually in-es the development of arteriolosclerosis. Furthermore, chronic hypoxia commonly encountered with onic ischemia and altered hemorrheology in glomerular microcirculation would have impact on the elopment of afferent arteriolar disease as recently proposed by Johnson and Mazzali [33].

he therapeutic benefit with combined formula observed in this study as well as others [34–37] sup-ts such conceptual view. Reduction in renal arteriolar resistances with vasodilators is followed by a re-tion in intraglomerular hydrostatic pressure and improvement in peritubular capillary flow (Table 2). e hemodynamic improvement coincides with the improvement in renal function namely glomerular ation rate or creatinine clearance, FE Mg and urine protein. It is of notion that the therapeutic benefit uires a much higher dose and longer duration of multi-drug vasodilators than needed for maximal od pressure control because of the sustainedly elevated efferent arteriolar resistance despite the blood ssure has already been converted back to normal (Table 2). The reason for using combined vasodila-s (angiotensin converting enzyme inhibitor, angiotensin II receptor antagonist and calcium channel cker) in treating these FSGS patients has been based on the previous therapeutic failure with the sin-drug therapy in reducing the efferent arteriolar resistance and restoring the creatinine clearance above pre-treatment value. Dipyridamole and baby aspirin ± heparin have been added to the therapeutic imen since the drugs can prevent further platelet consumption and intravascular clotting process.

Recently, Komine et al. [38] and Remuzzi et al. [39] have demonstrated an interesting observation t vasodilators such as AII receptor antagonist and ACEI can suppress angiotensin II formation in the ney and suppress the inflammation and profibrotic process in the kidney in animal model. The decline FE Mg which correlates directly with the magnitude of tubulointerstitial fibrosis [5] and *the improve-nt in* peritubular capillary flow which correlates inversely with the decline in FE Mg [40] observed this study following therapy renders support to the preceding view of inflammatory regression and proved flow in microcirculation by vasodilators.

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Glomerular endothelial cytotoxity and dysfunction in nephrosis with focal segmental glomerulosclerosis

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Abstract. Glomerular endothelial cell dysfunction (GED) with defective release of vasodilator has been delineated in nephrosis (NS) *in vivo* and *in vitro* studies. In NS with focal segmental glomerulosclerosis (FSGS), an immunocirculatory balance may be impaired due to defective anti-inflammatory cytokine. This study aimed at simultaneous determination of both proinflammatory cytokine (tumor necrosis factor alpha) and an anti-inflammatory cytokine (interleukin-10) in NS with FSGS. An endothelial cell cytotoxicity (ECC) was also examined using nephrotic serum. It was shown that (1) the initial endothelial cell cytotoxicity was significantly different from the control, (2) ratio between tumor necrosis alpha and interleukin-10 was significantly elevated, and (3) intrarenal hemodynamics was changed significantly.

Keywords: Glomerular endothelial dysfunction, cytokine, endothelial cell cytotoxicity, nephrosis, focal segmental glomerulosclerosis

1. Introduction

A normal glomerular endothelial function expresses an anticoagulant surface due to the property of negatively charged surface as well as releases an adequate amount of vasodilators namely prostacyclin and nitric oxide thereby it allows a free-flowing of non-coagulant blood. In addition, the negatively charged surface prevents the leakage of serum protein into the urinary space [1].

In this regard, the presence of proteinuria observed in nephrotic patient implies that the glomerular endothelial function is defective. A study in an experimental model of nephrosis in animal induced by aminonucleoside nephrosis reveals that the induction of proteinuria is associated with the loss of negatively surfaced charge [2].

The loss of negatively surfaced charge can be induced by multiple factors such as metabolic, immunologic and hemodynamic triggers. A metabolic product such as reactive oxygen species which is generally enhanced in nephrosis can induce injury to the glomerular endothelial cell [3]. It has also been earlier suggested by Shalhoub [4] that immunologic mechanism such as T cell disorder is associated with minimal change disease. Subsequently, Lagrue [5] and Saxena [6] denoted that there was a high level of interleukin-2 in cultured supernatant fluid of activated lymphocyte from minimal change disease.

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Koyama [7] found that T cell hybridoma from minimal change disease produced a glomerular permeability factor with its property similar to tumor necrosis factor alpha. Bricio [8] also demonstrated an elevated level of interleukin-1 in cultured whole glomeruli obtained from rats with adriamycin induced nephrosis. Recently, Matsumoto [9,10] denoted an augmented level of interleukin-12 and interleukin-18 in minimal change disease.

The preceding information renders support that the proinflammatory cytokines of T helper 1 is involved in the mechanism of proteinuria. However, the issue concerning the anti-inflammatory cytokine of T helper 2 is completely lacking. It is the purpose of the present study to perform the simultaneous determination of proinflammatory cytokine of T helper 1 (tumor necrosis factor alpha) and of anti-inflammatory cytokine of T helper 2 (interleukin-10) in nephrosis associated with focal segmental glomerulosclerosis (FSGS).

One of the most interesting issue observed in nephrosis associated with FSGS is the progressive reduction in renal plasma flow as the disease severity progresses [11]. This would raise a question whether there should be certain toxic substance in the serum that is capable of inducing such phenomenon of spontaneous reduction in renal perfusion. Therefore, we also examined an endothelial cell cytotoxicity (ECC) using nephrotic serum.

2. Material and method

10 patients associated with FSGS and 10 normal healthy controls with similar mean age were included for the following studies

2.1. Endothelial cell cytotoxicity (ECC test)

We performed an ECC test using sera from nephrotic patients as previously described [12]. In brief, the human endothelial cell line ECV 304 (American Tissue Culture Collection) in medium 199 with 10% fetal bovine serum. Approximately 2×10^4 cells/well of 96-well tissue culture plates was incubated overnight at 37°C in a 5% CO_2 atmosphere. Sera from nephrotic patients were added in duplicate wells. The culture medium and 10% Triton X were used as controls that showed no cell lysis and 100% cell lysis, respectively. The testing cultures were incubated as above for an additional 48 h. After incubation, each well was washed with phosphate-buffered saline and then stained with crystal violet. The stained cells were lysed with acid alcohol solution, and the optical density (OD) was determined by using a microtiter plate reader (moded 3550, Biorad) at 550 nm. The percentage of cytotoxicity was calculated by:

$$\text{Percent cytotoxicity} = 1 - \frac{\text{OD}_{\text{testing}} - \text{OD}_{\text{TritonX}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{TritonX}}} \times 100\ (\%).$$

2.2. Cytokine study

Sera from nephrotic patients were subject to the study of tumor necrosis factor alpha representing proinflammatory cytokine of T helper 1 and of interleukin-10 representing the anti-inflammatory cytokine using the commercial kit as previously described [13].

2.3. Intrarenal hemodynamic study [14]

Simultaneous assessment of effective renal plasma flow (RPF) using ^{131}I-labelled orthoiodohippuric acid (hippuran) and of glomerular filtration rate (GFR) using ^{99m}Tc-labelled DTPA were determined. Intrarenal hemodynamics were calculated and based on modified Gomez's equation. For calculation purpose, the effective filtration pressure across the glomerular capillaries (PF) is assumed to be 35 mm Hg when the blood pressure is normal (BP 120/80) mm Hg or less) and 40 mm Hg when the blood pressure is high (BP > 120/80 mm Hg). The hydrostatic pressure in Bowman's space (Ht) is assumed to be 10 mm Hg, the RPF and GFR are in ml/sec/1.73 m^2, the RBF is RPF/(100 − hematocrit) × 100. From the above assumptions, the following equations are derived:

$$\textbf{\textit{the intraglomerular hydrostatic pressure (PG)}} = \mathrm{PF} + \mathrm{Ht} + 5\left(\frac{\mathrm{TP}}{\mathrm{FF}} \times \log \frac{1}{1-\mathrm{FF}} - 2\right),$$

where TP and FF are plasma total protein in gram and filtration fraction, respectively, as follows:

$$\textbf{\textit{efferent arteriolar resistance (RE)}} = \frac{\mathrm{PF} \times 1328}{\mathrm{RBF} - \mathrm{GFR}}.$$

2.4. Statistical analysis

Values in text are expressed as mean ± SEM. Non-parametric ANOVA was used to establish the significance of between group difference. The difference was statistically significant when the p value was less than 0.05.

3. Results

3.1. Endothelial cell cytotoxicity test

The initial endothelial cell cytotoxicity was 35 ± 9% (normal 0.6 ± 1.2%) which was significantly different from the control; $p < 0.001$.

3.2. Cytokine study

The result of the study indicated that there was a significant elevation of tumor necrosis factor alpha (8.8 versus 3.3 pg/ml of normal control; $p < 0.05$) and a non significant change in interleukin-10 (2.3 versus normal 2.3 pg/ml) and the ratio between tumor necrosis alpha and interleukin-10 was 3.9 pg/ml versus 2.5 pg/ml of normal control which was significantly elevated $p < 0.01$.

3.3. Intrarenal hemodynamics

A significant change in intrarenal hemodynamic study was delineated as follows. The GFR was 34 ± 13 ml/min/1.73 m^2 versus control of 116 ± 16 ml/min/1.73 m^2; $p < 0.001$. The RPF was 178 ± 73 ml/min/1.73 m^2 versus control of 595 ± 45 ml/min/1.73 m^2; $p < 0.001$. The peritubular capillary flow (PTCF) was 144 ± 67 ml/min/1.73 m^2 versus 480 ± 46 ml/min/1.73 m^2 of control; $p < 0.001$. The intraglomerular hydrostatic pressure (PG) was 55 ± 2 mm Hg versus 51 ± 0 mm Hg of control; $p < 0.01$. The efferent arteriolar resistance (RE) was 15648 ± 8538 dyn.s.cm^{-5} versus 3062 ± 177 dyn.s.cm^{-5} of control; $p < 0.001$.

4. Discussion

An abnormal immunocirculatory balance has been delineated and is reflected by both an elevated tumor necrosis factor alpha and a defective interleukin-10 in nephrosis associated with FSGS. Such an observation has recently been supported by Lama and associates. They demonstrated an enhanced proinflammatory cytokine (tumor necrosis factor alpha) and a defective anti-inflammatory cytokine (interleukin-10) in cultured supernatant from T lymphocytes in nephrosis with FSGS [15]. This immunocirculatory imbalance is likely to induce a sustained proinflammatory cytokine effect in nephrosis with FSGS by which it would explain the clinical characteristically sustained proteinuria.

The immunocirculatory disturbance would exert a significant impact upon the glomerular endothelial function. Under such circumstance, a toxic trigger can sustainedly injure the glomerular endothelial structure and function. Indeed, glomerular endothelial cytotoxicity and dysfunction has been substantiated. The glomerular endothelial cytotoxicity may be greatly enhanced by adding nephrotic serum from FSGS. In human clinical setting, an endothelial cell of glomerular as well as postglomerular microcirculation of nephrosis with FSGS may be significantly depleted by means of endothelial factor VIII staining method. In respect to the glomerular endothelial function observed in nephrosis with FSGS, a dysfunctioning glomerular endothelium is reflected by: (i) the loss of negatively charged surface which concomittently induces proteinuria and activates the local intravascular coagulation, (ii) the alteration in intrarenal hemodynamics which is due to the defective release of vasodilator.

In accordance with the study, a five-fold increase in efferent arteriolar resistance is observed. Such a preferential constriction at the efferent arteriole exerts 2 significant hemodynamic impacts. Proximal to the efferent arteriolar constriction, it induces intraglomerular hypertension which correlates with the incidence of glomerulosclerosis [16]. Distal to the efferent arteriolar constriction, it exaggeratedly reduces the peritubular capillary flow. A three-fold decrease in peritubular capillary flow is documented in this group of patient. This magnitude of reduction in renal perfusion would induce ischemic injury to the tubulointerstitial structure and eventually the development of tubulointerstitial fibrosis.

The preceding information supports the conceptual view that the greater magnitude of glomerular endothelial dysfunction in nephrosis with FSGS is in accordance with an underlying immunocirculatory disturbance. Improvement in glomerular endothelial cell cytotoxicity and dysfunction can be accomplished with vasodilator, antioxidant and herbal medicine [17].

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GLOMERULAR ENDOTHELIAL DYSFUNCTION AND MICROVASCULAR DISORDER IN CHRONIC KIDNEY DISEASE

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Introduction

Glomerular endothelial dysfunction and microvascular disease are the crucial determinants of renal disease progression, and failure to recognize such disorders is responsible for the present therapeutic failure in preventing the progression to end stage renal disease. In this regard, Bohle was the first to demonstrate an inverse correlation between the relative area of cortical widthness and the postglomerular capillary patency[1]. Similar observation was also noted by Yenrudi an inverse correlation between peritubular capillary flow reduction and the renal cortical pathology in patients with nephrotic syndrome[2]. Loss of microvascular endothelium was also demonstrated which correlated with renal disease severity[3,4]. Since renal microvascular disease is often diagnosed when the renal tissue injury has already been advanced, a search for biomarkers that would early reflect microvascular disease is warranted.

Material and Method

1. One volume of 0.019 M trisodium citrate solution was mixed with 9 volumes of blood and the mixture was centrifuged at 2500 x g for 10 min to collect plasma. The citrated plasma was stored at –20°C for determination of plasma factors. Three milliliters of EDTA blood was collected for isolation of circulating endothelial cells by method described by Solovey[5]. Determinations of sVCAM-1, vascular endothelial growth factor (vEGF), transforming growth factor beta were performed by using commercial ELISA kits.
2. Intrarenal hemodynamic study.

Simultaneous assessments of effective renal plasma flow (RPF) using ^{131}I-labeled orthoiodohippuric acid (hippuran) and of glomerular filtration rate (GFR) using ^{99m}Tc-labelled DTPA were determined by the previously described method[6].

3. Tubular function was assessed by determining the fractional excretion of magnesium (FE Mg) as previously described[7].

Results

In chronic kidney diseases, elevated levels of circulating endothelial cells (2169±1336 vs control 59±57 cells/ml; p <0.01), enhanced transforming growth factor beta (4718± 2246 vs control 2014±794 pg/ml; p NS), and sVCAM (531±186 vs control 38685 ng/ml; p NS), and depleted levels of VEGF (74±57 vs control 548±360 pg/ml; p <0.01) were documented. Intrarenal hemodynamics revealed (i) significant reductions of renal plasma flow (234±97 vs control 589±41 ml/min/1.73m^2), peritubular capillary flow (182±81 vs control 483±43 ml/min/1.73m^2; p <0.001), glomerular filtration rate (51±23 vs control 114±14 ml/min/1.73m^2; p <0.01). (ii) significant elevations of intraglomerular pressure (55±2 vs control 51±0.02 mm Hg; p <0.001), afferent arteriolar resistance (10753±6426 vs control 2442±138 dyne.s.cm^{-5} and efferent arteriolar resistance (12447±7082 vs control 3042±165 dyne.s.cm^{-5}; p <0.01). Tubular function by mean of FE Mg showed abnormally elevated (6.3±2 vs 1.6±0.6%; p <0.001).

Discussion

Since blood circulation contains toxins such as oxidative stress and proinflammatory cytokine that can easily gain access to glomerular capillary, the glomerular capillary is likely to be the primary site of kidney inflammation. Our study tends to support this conceptual view. Increased number of circulating endothelial cells reflects the enhancement of endothelial cell loss which correlates with the depletion of VEGF and increased in-vitro endothelial cell cytotoxicity observed in these patients with chronic kidney diseases [8]. Enhanced transforming growth factor beta may be partly responsible for the VEGF reduction since it is capable of inducing apoptosis of podocyte and tubular epithelium which are the main source of VEGF production[9].

Enhanced circulating endothelial cell loss and VEGF depletion are early biomarkers of glomerular endothelial cell (GEC) injury.

GEC injury correlates with glomerular endothelial dysfunction determined by intrarenal hemodynamic study. An altered intrarenal hemodynamics uniquely observed in chronic kidney diseases is characterized by hemodynamic maladjustment with preferential constriction of the efferent arteriole[10,11]. Such constriction induces **proximally** an intraglomerular hypertension, capillary dilation with subsequent podocyte loss and VEGF depletion, and **distally** peritubular capillary flow reduction. The reduction in peritubular capillary flow precedes the development of tubulointerstitial fibrosis (TIF) and correlates inversely with TIF, therefore it signifies the cause-and-effect relationship[12]. It is also noted that the magnitude of TIF correlates directly with fractional excretion of magnesium (FE Mg)[7]. **Practically, FE Mg is an early marker for screening of renal disease severity.**

With respect to the therapeutic strategy, a successful therapeutic intervention can be accomplished by (1) correcting the hemodynamic maladjustment with multidrug vasodilators and (2) early screening of microvascular injury with circulating endothelial cells, VEGF or early screening of the disease severity with FE Mg.

Key words : Microvascular disease, Hemodynamic maladjustment, Chronic kidney disease. Circulating endothelial cell, FE Mg.

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CLINICAL STUDY

Biomarkers of Endothelial Injury in Focal Segmental Glomerulosclerotic Nephrosis

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Enhanced circulating endothelial cells, elevated transforming growth factor beta, and depleted vascular endothelial growth factor were observed in nephrosis associated with focal segmental glomerulosclerosis (FSGS). Increased endothelial cell loss may be due to the elevated transforming growth factor beta, which can induce apoptosis of podocyte as well as tubular epithelium. Such injury may explain the depletion of vascular endothelial growth factor and increased endothelial cell loss in these patients. There biomarkers may have relevance to the altered intrarenal hemodynamics commonly observed in FSGS nephrosis.

Keywords circulating endothelial cell, hemodynamics, transforming growth factor beta, vascular endothelial growth factor, nephrosis

INTRODUCTION

Glomerular endothelial cell dysfunction and renal microvascular injury have previously been noted in nephrosis associated with focal segmental glomerulosclerosis (FSGS). These are reflected by an enhanced endothelial cell cytotoxicity induced in vitro by a patient's own serum[1] and by a decreased endothelial factor VIII staining in the renal microcirculation of FSGS nephrosis,[2] respectively. Such glomerular endothelial cell dysfunction can be assessed by intrarenal hemodynamic study that usually reveals a reduction in renal plasma flow and peritubular capillary flow which supplies the tubulointerstitium.[3,4] The reduction in peritubular capillary flow has therapeutic implications, because peritubular capillary flow reduction is relevant to the pathogenesis of tubulointerstitial fibrosis and renal disease progression.[5] Early detection of glomerular endothelial cell injury or dysfunction would have better impact on the clinical response to therapy than the late recognition of the vascular disease. In this regard, determination of intrarenal hemodynamic study is generally not available due to limited resource, therefore, a search for an alternative biomarker that would reflect endothelial cell injury would have relevance to the therapeutic strategy. It is the purpose of this study to perform biomarkers that would reflect endothelial cell injury, such as circulating endothelial cell, transforming growth factor beta, and vascular endothelial growth factor in nephrotic patients associated with FSGS.

MATERIAL AND METHOD

Fifteen nephrotic patients associated with FSGS were enrolled in the study. Ten age-match subjects served as healthy controls.

Enumeration of Circulating Endothelial Cells

The number of circulating endothelial cells was examined using the buffy coat smear technique as

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described by Solovey.[6] Diluted blood was layered on a Ficoll-Hypaque density gradient (Histopaque-1077, Sigma) and sedimented for 30 min at 250 g. The pooled supernatant and interface were centrifuged for 5 min at 1200 g, and a smear was made of buffy-coated pellet. After being air dried, the smear was fixed with 4% paraformaldehyde for 10 min and stained with FITC-conjugated sheep antihuman von Willebrand factor (Seroter, UK). The number of circulating endothelial cells per 1 ml of whole blood was examined using fluorescence microscopy. Negative controls were provided by the white cells on the smears and by parallel slides prepared with control negative antibody.

ELISA for Vascular Endothelial Growth Factor (VEGF)

This assay employed the quantitative sandwich enzyme immunoassay technique. Standards and samples were pipetted into the wells, and any VEGF present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. Following a wash to remove any unbound antibody enzyme reagent, a substance solution was added to the wells, and color developed in proportion to the amount of VEGF bound in the initial step. The color development was stopped, and the intensity of the color was measured.

ELISA for Transforming Growth Beta (TGF-β)

TGF-β-soluble receptor type II, which bound TGF-β1, had been precoated onto a microplate. Standards and samples were pipetted into the wells, and any TGF-β1 present was bound by the immobilized receptor. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-β1 was added to the wells to sandwich the TGF-β1 immobilized during the first incubation. Following a wash to remove any unbound-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of TGF-β1 bound in the initial step. The color development was stopped, and the intensity of the color was measured.

STATISTICAL ANALYSIS

Comparison of the sample mean of two quantitative variables was determined by the nonparametric method using the Mann–Whitney test. The difference between groups was performed by Student's unpaired *t*-test. The *p* values below 0.05 were considered to be significant.

Table 1
Biomarkers of endothelial injury in FSGS nephrosis

	Circulating-endothelial cell (cell/mL)	TGF β (pg/mL)	VEGF (pg/mL)
I Control	46±67	3224±712	299±150
p value	<0.001	<0.001	<0.05
II FSG nephrosis	2528±585	7796±2400	64±50

RESULTS

Table 1 showed a significantly elevated number of circulating endothelial cells, transforming growth factor beta, and depleted level of vascular endothelial growth factor.

DISCUSSION

By virtue of its location facing the circulating blood and toxic elements such as oxidative stress, proinflammatory cytokine, and toxins, the glomerular endothelial cell is likely to be injured by such substances and is generally the primary site where kidney inflammation takes place.[7] The result of our study tends to support this conceptual view. An increased number of circulating endothelial cells was noted in FSGS nephrosis. This finding implies enhanced endothelial cell loss. Glomerular endothelial cell dysfunction,[8] namely, charges selective proteinuria, a defective release of endothelium-dependent vasodilators, which has previously been substantiated in nephrotic syndrome, concurs with the increased endothelial cell loss. The increased production of TGF-β observed in these nephrotic patients may be relevant to the mechanism of endothelial cell loss and suppression of VEGF production, because TGF-β can induce apoptosis of podocyte[9] as well as tubular epithelium,[10] which are the main sources of VEGF production. In addition, depleted VEGF production may be due to enhanced detachment of podocyte from the basement membrane secondary to hemodynamic maladjustment.[3] VEGF production was noted to be depleted (Table 1). Because VEGF is a crucial growth factor that is essential to the survival and regeneration of endothelial

cells, depletion of VEGF may be relevant to enhanced endothelial cell loss.

In accordance with the preceding information, an increased number of circulating endothelial cells may be a suitable marker that reflects renal microvascular disease. Accumulating evidence indicates that renal microvascular disease is crucial to the development of nephronal damage.[2,11–13] Early detection of renal microvascular disease or glomerular endothelial cell injury by such a marker would be beneficial to the preventive strategy of progressive renal disease.

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