ORIGINAL ARTICLES

Decreased synthesis of glomerular adrenomedullin in patients with IgA nephropathy

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Adrenomedullin (AM) immunostaining and gene expression have seldom been measured in human kidneys. Because previous studies have shown that AM exerts antiproliferative effects on rat mesangial cells in vitro and that urine AM levels are decreased in patients with chronic glomerulonephritis, we measured glomerular AM and its gene expression in patients with primary IgA nephropathy (IgAN). Glomerular AM was measured by immunohistochemical staining, and glomerular AM mRNA was measured by in situ hybridization. Plasma and urine AM were measured by radioimmunoassay. The results showed that both the intensity of immunostaining for glomerular AM and the glomerular expression of AM mRNA were significantly decreased in IgAN patients compared with normal controls (both $P < .05$). Similar results were not observed in patients with non-IgA MsPGN. Glomerular AM immunostaining and glomerular AM mRNA expression were significantly correlated ($P < .001$), and both were negatively correlated with the number of glomerular cells ($P < .05$ and $< .01$, respectively). Both glomerular AM immunostaining and glomerular AM mRNA expression were correlated with urine AM levels (both $P < .001$), but not with plasma AM levels. The urine AM level was significantly lower in IgAN patients than in normal controls ($P < .01$), whereas the plasma level was not different between the 2 groups. Our findings indicate that glomerular production of AM was decreased in patients with IgA nephropathy and that this lack of glomerular AM may be related to the pathogenesis of this mesangial disease. (J Lab Clin Med 2005;145:233–238)

Abbreviations: AM = adrenomedullin; CCr = creatinine clearance rate; cDNA = complementary DNA; IgAN = IgA nephropathy; MsPGN = mesangial proliferative glomerulonephritis; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; RIA = radioimmunoassay; SSC = standard saline citrate

IgA nephropathy (IgAN) is characterized by the predominance of IgA deposition in the glomerular mesangium and is now recognized as the most common glomerulonephritis worldwide.[1] Despite intensive investigations by several groups, a widely accepted hypothesis for the pathogenesis of IgAN has not emerged. Recent studies have revealed increased plasma levels and decreased urine levels of adrenomedullin (AM) in patients with IgA nephropathy,[2] indicating that AM may be involved in the pathogenesis of this disease.

AM is a hypotensive peptide originally isolated from an extract of pheochromocytoma tissue.[3] AM peptides consist of 52 amino acids in human and 50 amino acids in rats. These peptides have a potent and long-lasting hypotensive effect when injected into animals.[4] Later studies have demonstrated that AM is synthesized not only in the adrenal gland, but also in the heart, lung, and kidney.[5] AM is a potent vasorelaxant, and there is increasing evidence that AM may function in a para-

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Table I. PCR primer name, sequence, and product size

Name	Primer sequence (5′ to 3′)	Size (bp)
AM (sense)	2435CGTCGGAGTTTCGAAAGAAG2454	817
AM (antisense)	3252CGTGTGCTTGTGGCTTAGAA3233	
T7+AM (sense)	ACTCACTATAGGGAGACGTCGGAGTTTCGAAAGAAG	833
T7+AM (antisense)	ACTCACTATAGGGAGACGTGTGCTTGTGGCTTAGAA	
T7U	TAAGCTTAAATACGACTCACTATAGGGAGA	466

crine and/or autocrine manner in the regulation of cardiovascular homeostasis.[6,7] AM immunostaining and gene expression have been detected in the kidneys of rat and dog,[8,9] and a significant level of AM has been detected in human plasma and urine by specific radioimmunoassay.[10] However, there have been only a few reports on AM immunostaining and AM mRNA expression in human kidneys.

Recent studies indicate that AM suppresses mitogenesis of rat mesangial cells.[11,12] Through its antiproliferative and antiapoptotic effects on the mesangial cells, AM may play a major role in the normal turnover of mesangial cells.[13] Because most patients with IgAN show a proliferation of glomerular mesangial cells, and because intraglomerular hypertension is common in patients with chronic glomerular diseases, it could be speculated that glomerular synthesis of AM is decreased, thus resulting in cellular proliferation and the loss of AM's vasodilating effects. To test this hypothesis, we measured glomerular AM staining in primary IgAN patients by immunohistochemical methods and glomerular AM mRNA expression by in situ hybridization. Both plasma and urine levels of AM were also measured.

METHODS

Patient selection. Glomerular AM was measured in 16 patients (10 males and 6 females; mean age 27 ± 6 years) with a clinical and pathological diagnosis of primary IgAN. All patients had normal renal function; their mean serum creatinine level was 0.9 ± 0.2 mg/dL and mean creatinine clearance rate (CCr) was 92 ± 11 mL/min. The mean daily protein loss was 0.82 ± 0.35 g. All patients were clinically quiescent and had been free from infection and macroscopic hematuria for at least 8 weeks. All patients gave informed consent, and none was receiving corticosteroids, immunosuppressive drugs, or angiotensin II converting enzyme inhibitors. Control kidneys were obtained from 12 patients with non-IgA mesangial proliferative glomerulonephritis (MsPGN)(8 males and 4 females; mean age 29 ± 4 years). Another 5 kidneys were obtained from patients who underwent nephrectomy because of renal cancer; these served as normal controls.

We also measured the urine and plasma levels of AM in those patients with IgAN and MsPGN. Patients with hypertension were not included in our study, because hypertension itself is associated with increased plasma AM levels.[14] Urine and sera from 15 apparently normal persons (8 males and 7 females; mean age 29 ± 5 years) were collected as controls.

Tissue processing and calculation of mesangial cells. Human kidney tissue was fixed in 10% buffered formalin and embedded in paraffin. A set of 3-μ-thick sections was stained with periodic acid-Schiff and hematoxylin and eosin. Light microscope examination of all patients showed a proliferation of glomerular mesangial cells, and total glomerular cells were counted in 25 glomeruli.

Immunohistochemical studies. Avidin-biotin-peroxidase complex staining method was used for the immunohistochemical study.[15] The tissue sections were first deparaffinated in xylene and then subsequently washed with 100%, 95%, and 70% ethanol, followed by a rinsing with distilled water. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol, followed by a wash with standard phosphate-buffered saline (PBS). The tissues were then blocked with 5% normal serum albumin using the Vectastatin ABC/DAB Elite Kit (mouse IgG type; Vector Laboratories, Burlingame, CA). Excess serum was blotted. A panel of primary antibodies specific for human AM (Peninsula Laboratories, Belmont, CA) was used. The tissues were washed in PBS and incubated with biotinyl-conjugated secondary antibodies (Vector Laboratories) in an immunostaining kit for primary antibody. Sections were then observed with an optical photomicroscope (Olympus, Japan). Glomerular staining was graded in each glomerulus semiquantitatively, and each was scored from 0 to 4+ according to the staining intensity (0 = 0, 1+ = 1, 2+ = 2, 3+ = 3, and 4+ = 4). We counted 25 glomeruli to get the final score, with a range of 0–100 for each patient.

Complementary RNA (cDNA) Probes. To generate T7 promoter-tailed AM DNA templates for in vitro transcription for AM cRNA probes, 2 successive polymerase chain reactions (PCRs) were performed as described by Cone et al.[16] In the first PCR, 2 separate amplifications for 35 cycles under standard conditions were performed using the AM (sense)/T7+AM (antisense) or T7+AM (sense)/AM (antisense) primer pairs. For each primer pair, 1 primer had no additional nucleotides (AM sequence only), and the other primer was tailed with the 3′ end of the T7 promoter. The products of the 2 reactions were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining. The first set of PCR products was purified with a purification kit (Roche Molecular Biochemicals, Mannheim, Germany). Then 5 μL of a 1:100 dilution of the first PCR products was reamplified using the AM primer as used in the first round of PCR and another composite primer (universal T7) (Table I), to extend and