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The possible role of granulocyte elastase in renal damage from acute pyelonephritis

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Abstract. During acute inflammatory processes, extracellular release of granulocyte elastase can contribute to subsequent tissue damage. To test our hypothesis that extracellular elastase release during acute pyelonephritis may contribute to subsequent renal parenchymal damage, we compared the intracellular and extracellular activities of the lysozyme elastase of human polymorphonuclear cells (PMN) when incubated in vitro with bacterial strains causing renal infection that led to either renal damage or no damage. Urine bacterial cultures were obtained from patients with acute pyelonephritis (flank pain, costovertebral angle tenderness, fever $>38°$ C, bacteriuria, pyuria, and leukocytosis). Renal damage was demonstrated by cortical scarring on followup intravenous pyelography and/or diminished function on ^{131}iodine hippuran renal scan. Mean extracellular elastase activity (μunits/PMN) was 0.15 for unstimulated PMN, 0.07 for PMN stimulated by bacteria not associated with renal damage, and 1.20 for the PMN stimulated by strains associated with renal damage. Mean intracellular elastase activity (μunits/PMN) was 3.73 for unstimulated PMN, 3.48 for PMN stimulated by bacteria not associated with renal damage, and 3.31 for the PMN stimulated by strains associated with renal damage. Extracellular granulocyte elastase activity was thus significantly higher ($P = 0.0001$) in PMN stimulated by bacterial strains associated with renal damage. Extracellular release of elastase may contribute to the pathogenesis of renal damage in pyelonephritis.

Key words: Pyelonephritis – Respiratory burst – Superoxide – Lysozyme – Elastase – Vesicoureteral reflux

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Introduction

Loss of renal function with cortical scar formation is the most important sequala of acute and chronic pyelonephritis, and its prevention has been the impetus for much investigation. One critical goal of research in the area of pyelonephritis is to identify risk factors for renal scarring associated with bacterial virulence factors and/or the elicited host inflammatory response.

The polymorphonuclear neutrophil (PMN) is a non-dividing short-lived white blood cell capable of engulfment and digestion of microorganisms [1]. It contains lysosomal granules which fuse with the phagocytic vacuole to form a phagolysosome which stimulates a concerted destruction of the ingested microbe by oxygen free radicals (superoxide, hydrogen peroxide, myeloperoxidase-generated halide ions, and singlet oxygen) and lysosomal enzymes. Some extracellular release of lysosomal constituents occurs, which stimulates an acute inflammatory reaction with increased vascular permeability leading to transudation of serum proteins and infiltration of PMN into adjacent tissue.

A correlation has been found between the degree of neutrophilic infiltration during acute pyelonephritis and the resultant renal scarring [2]. Granulocyte depletion with cyclophosphamide and prevention of PMN migration with colchicine prevent renal tissue damage [3]. Prevention of renal scarring requires antibacterial treatment within 28 h to prevent early influx of PMN and acute suppuration [3].

Human granulocyte (leukocyte) elastase is a glycosylated, highly basic serine protease contained in the azurophilic granules of the polymorphonuclear cell [4]. Elastase has been demonstrated to play a major physiological role in the digestion of phagocytosed *Escherichia coli* proteins by human PMN, as lysosomal degradation of *E. coli* proteins was significantly inhibited when elastase activity was specifically inhibited [5].

Extracellular release of elastase, however, may contribute to tissue damage associated with inflammatory processes because of its neutral optimum pH and its broad spectrum of proteolytic activity (substrates include collagen IV, laminin, and proteoglycans). Granulocyte elastase ac-

Table 1. Patient details and bacterial virulence factors

Age	Sex	Bacteria	P-fimbriae	Type 1	Hemolysin	Notes			Reflux
						Patients with renal damage			
8	M	JR649	Neg	Pos	Neg	QRS	R61%	L39%	Yes
4	F	BK309	Neg	Pos	Neg	IVP	Cortical atrophy	L. lower pole	Yes
6	F	JR352	Pos	Pos	Neg	QRS	R40%	L60%	Yes
						Patients with no renal damage			
8 months	F	BK228	Neg	Pos	Neg	IVP and QRS		Normal	No
6	F	BK142	Pos	Pos	Pos	QRS		Normal	No
3	F	BK137	Neg	Pos	Neg	IVP		Normal	No
10 months	F	JR302	Neg	Pos	Neg	IVP and QRS		Normal	Yes
17	F	JR389	Neg	Pos	Neg	IVP		Normal	No

QRS, Quantitative renal scan; IVP, intravenous pyelogram

tivity is increased in inflammatory tissue and fluid from patients with adult respiratory distress syndrome, pneumonia, rheumatoid arthritis, and burn injuries [6]. A balance exists between extracellular elastase and its endogenous inhibitors, α_1-proteinase inhibitor and secretory leukocyte protease inhibitor; however, in disease states this balance may be disrupted [7]. The α_1-proteinase inhibitor is an acute-phase reactant product of the hepatocyte that can increase fourfold during stress [8].

We hypothesized that extracellular elastase release during acute pyelonephritis may contribute to subsequent renal parenchymal damage. This study compares the intracellular and extracellular granulocyte elastase activities of isolated human polymorphonuclear cells when incubated with bacterial strains isolated from patients suffering from pyelonephritis with and without renal damage.

Materials and methods

Patients. Details of patients and bacterial virulence factors are presented in Table I. Acute pyelonephritis was defined as flank pain with costovertebral angle tenderness, fever (>38° C), bacteriuria, pyuria, and leukocytosis. Flank pain and costovertebral angle tenderness were not used as inclusion criteria in the two infants. Note that four of the patients had vesicoureteral reflux (low grade and non-dilating) as a complicating factor. Renal scarring was demonstrated by cortical and medullary lesions overlying dilated calyces on followup intravenous pyelography and loss of renal function was determined using ^{131}iodine hippuran quantitative renal scan. These studies were performed on followup of either the first febrile urinary tract infection in the patient or previous radiographic studies had demonstrated no renal scarring; allowing us to attribute any ensuing renal damage to the current infectious organism. The validity and reproducibility of quantitative renal scan to assess renal function and damage has previously been established in the primate model [9].

Percoll solution preparation. Percoll stock (100%) was prepared by combining 9 volumes of Percoll (Pharmacia, density 1.131 g/ml) with 1 volume of 10 times Hanks' balanced salt solution (HBSS) lacking calcium and magnesium (Gibco). Stock was diluted to 81%, 70%, or 55% (v/v) with 1 times HBSS containing 10 mM Hepes buffer (Gibco, pH 7.3). Densities of these solutions were 1.1000, 1.0875, and 1.0697 g/ml, respectively.

PMN isolation. PMN isolation was performed using Percoll density gradient centrifugation [10]. Whole blood from healthy volunteers was collected in heparinized tubes and pooled. Buffy coat was obtained by centrifugation (3,750 *g*, 3.25 min) in a swing-out rotor. An equal volume of 4% dextran in phosphate-buffered saline (PBS) was added to yield a 2% dextran cell suspension. After 6 min, the volume was again doubled (total of 4 times initial volume) by the addition of 2% dextran in PBS, and red cells were allowed to settle in polypropylene centrifuge tubes for 30 min.

The red cell-depleted supernatant was removed and cells washed twice with PBS by centrifugation (150 *g*, 8 min). The cell pellet was then resuspended in 2 ml of 55% Percoll; 2.5 ml of 81% Percoll was pipetted into a polystryene tube to form the bottom shelf; 1.5 ml of 70% Percoll was overlaid, followed by the 2 ml of cell suspension in 55% Percoll. Gradient centrifugation was then performed (1,600 *g*, 20 min). The PMN-rich middle band was harvested by pipette suction. PMN extract (50 µl) was added to 2.45 ml of Turk's counting solution (0.01% gentian violet in 2% acetic acid). The cell count was performed using a Spencer Brightline hemocytometer and the PMN extract was diluted to a final concentration of 10^5 PMN/ml.

Bacterial isolation and preparation. The bacteria studied were originally obtained from patients with acute pyelonephritis and aliquots frozen for later study. Samples were then thawed and plated on sheep blood agar plates. Following a 24-h incubation at 37° C, isolated colonies were replated and incubated. API identification was performed on all isolates. Presence of type 1 fimbriae was detected by agglutination of 1% yeast (*Saccharomyces cerevisiae*) in the presence and absence of 30 mM D-mannose [11]. P-fimbriae was detected by the P particle agglutination kit (Swedish Institute for Infectious Disease Control, Stockholm, Sweden) [11]. Hemolytic activity was measured by inoculation of bacteria on blood agar made with sheep cells, followed by incubation at 37° C overnight to determine a clear area of hemolysis around the bacterial colonies [11]. Type 1 fimbriae, P-fimbriae, and hemolysin status are listed in Table 1. A final bacterial concentration of 10^9 was obtained by dilution with sterile normal saline, and confirmed by spectrophotometric density analysis.

PMN and bacterial incubation. All tests were performed in triplicate; 100 µl of PMN extract was incubated with 200 µl of bacterial suspension and 200 µl of KRG (Krebs-Ringers phosphate buffer + glucose 10 mM + calcium 1.0 mM + magnesium 1.5 mM, pH 8.3) at 37° C for 2 h (final concentrations 2×10^4 PMN/ml, 4×10^8 bacteria/ml). The mixture was centrifuged (500 *g*, 15 min) and 200 µl of supernatant removed for testing of extracellular elastase activity. Microscopic examination of the supernatant confirmed the absence of cells and bacteria. The cell pellet was resuspended in 200 µl of KRG for testing of intracellular elastase activity.

Granulocyte elastase assay. L-Pyroglutamyl-L-prolyl-L-valine-*p*-nitroanilide (S-2484, Kabi Pharmaceuticals) is a chromogenic substrate highly specific for ganulocyte elastase that is not hydrolyzed by pancreatic elastase, trypsin, and chymotrypsin [6, 12]. S-2484 stock solution (25 mg in 7 ml of dimethylsulfoxide, 8 mM) was diluted 1:3 with distilled water to yield a substrate concentration of 2 mM. Human leukocyte elastase (245 units/mg, Sigma) was serially diluted with TRIS-NaCl buffer (TRIS 0.1 M, NaCl 0.96 M, pH 8.3) and used as the initial test samples to create a standard curve of elastase activity

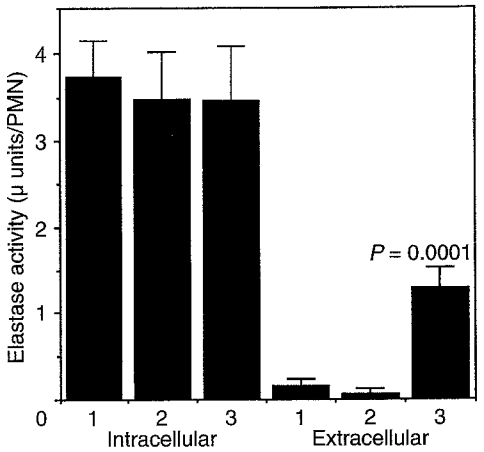

Fig. 1. Intracellular and extracellular elastase activities (μunits/PMN) following incubation of the polymorphonuclear neutrophils (PMN) with no bacteria (1), bacteria not associated with renal damage (2), and bacteria associated with renal damage (3). This demonstrates a significant increase in extracellular elastase activity when the PMN are stimulated with bacterial strains associated with renal damage

(units/l). TRIS-NaCl buffer, test sample, and S-2484 substrate (200 μl of each were incubated at 37° C for 3 h. Diazotization to enhance sensitivity was then performed, as previously reported, by the addition of 500 μl each of 5.8 mM sodium nitrite in 0.48 M hydrochloric acid, 26.3 mM ammonium sulfamate, and 2.7 mM *N*-1-napthyl-ethylenediamine dihydrochloride [6, 12]. Absorbance was then measured at 545 nm.

Statistical analysis. Data are expressed as mean plus or minus standard deviation. Student's *t*-test was used for all comparisons.

Results

No significant difference was found in mean intracellular elastase activity (μunits/PMN) of the unstimulated PMN ($3.73 \pm .41$), the PMN stimulated by bacteria not associated with renal damage ($3.48 \pm .54$), and the PMN stimulated by bacterial strains that were associated with renal damage ($3.31 \pm .89$). However, mean extracellular granulocyte elastase activity was significantly higher in PMN stimulated by the bacterial strains from patients with renal damage ($1.20 \pm .38$) than both unstimulated PMN ($0.15 \pm .09$) and PMN stimulated by strains not associated with renal damage ($0.07 \pm .05$) ($P = 0.0001$). The difference between PMN stimulated by strains without renal damage and unstimulated PMN was not significant (Fig. 1). Correlation with the presence of bacterial hemolysin was poor, as only one of the bacteria in each group carried the hemolysin. It is of importance that all of the patients with renal damage were compromised, with vesicoureteral reflux in addition to high extracellular elastase.

Discussion

Bacterial virulence factors and/or the elicited host inflammatory response may contribute to renal damage following acute pyelonephritis. In the present study both complicating host factors such as vesicoureteral reflux and the ability of some bacterial strains to affect the inflammatory response were found. The role of the neutrophil in the inflammatory process associated with pyelonephritis has been established. A direct correlation was demonstrated between the degree of acute-phase neutrophil infiltration and renal scar formation in a rat model of pyelonephritis [2]. Sustained-release corticosteroids initially modulated neutrophilic infiltration, however they subsequently reduced host defenses, resulting in bacterial proliferation and increased renal scar size [2, 3].

Complement-dependent opsonization of bacteria activates neutrophils in the tubular lumen via receptors for C3 on the neutrophil surface. A respiratory burst follows within the phagosome and on the phagocyte surface [13]. We have previously demonstrated in the primate model of pyelonephritis that intrarenal reflux of nephropathogenic strains of *E. coli* activates complement and chemotaxis within minutes [14, 15]. Within 48 h, up to 30% of renal function is lost due to renal tubular destruction by free oxygen radicals generated by the phagocytic respiratory burst. Intrarenal capillaries are blocked with leukocyte aggregrates within 60 min of inoculation, producing renal ischemia [14–16].

The extracellular component of the respiratory burst is larger when the response is induced by uropathogenic *E. coli* strains that cause renal scarring [17]. In the primate model of ascending acute pyelonephritis, exogenously administered parenteral superoxide dismutase was totally excreted in the urine, and decreased the extent of neutrophilic infiltration by inhibiting superoxide formation, and partially prevented renal tubular cell death and ultrastructural change [14].

Reactive oxygen species generated by stimulated PMN inactivate α_1-protease inhibitor by oxidation of a critical methionine residue which decreases its ability to inhibit elastase and increases susceptibility to cleavage by free elastase [8, 18, 19]. Hydroxyl radicals, superoxide radical, hydrogen peroxide, and myeloperoxidase-mediated reactions are involved in this mechanism, and α_1-proteinase inhibitor activity can be partially protected with superoxide dismutase, catalase, and mannitol [18, 19]. Activated neutrophils also release metalloproteinases that inactivate α_1-proteinase inhibitor [20].

Bacterial strains associated with renal damage have been shown to have an increased affinity for binding to neutrophils, which would augment the subsequent inflammatory response [17]. Type 1 fimbriae mediate interactions with the PMN that lead to adherence and phagocytosis and promote degranulation of PMN, probably due to a large hydrophobic surface area [21]. Type 1 fimbriated *E. coli* produced larger renal scars (by planimetric measurement) than P-fimbriated and non-fimbriated strains in a rat model, probably because the rat urothelium has receptors for type 1 fimbriae [21].

Granulocyte elastase plays a crucial role in bacterial digestion [5]. Extracellular release of elastase may contribute to the pathogenesis of renal scarring in pyelonephritis, as suggested by the greater extracellular release of elastase when we incubated bacteria from strains associated with renal damage. Elastase has been shown by in vitro studies to degrade collagen IV in the glomerular basement

membrane [22]. Topley et al. [21] demonstrated that non-specific lysosomal neutral protease activity was higher in PMN stimulated by type 1 fimbriated bacterial strains. All strains in our study possessed type 1 fimbriae, suggesting that additional virulence factors may play a role in determining the degree of extracellular elastase release.

Degranulation of azurophilic granules and release of extracellular myeloperoxidase and elastase could act in concert to destroy renal parenchyma, as myeloperoxidase-generated oxidants could inhibit α_1-proteinase inhibitor activity. Renal scarring was decreased in the rat model of acute pyelonephritis by treatment with dapsone to inhibit myeloperoxidase-mediated oxidation of halides [3]. Free oxygen radicals generated by macrophages and tissue-bound xanthine oxidase could also inhibit α_1-proteinase inhibitor [23]. Hypoxanthine is generated during the relative ischemia produced by a decrease in renal blood flow associated with infection. Xanthine oxidase metabolizes this to uric acid and superoxide during reperfusion [15]. We have previously demonstrated the ability of allopurinol, a xanthine oxidase inhibitor, to produce a dose-dependent decrease in tubular cell destruction [15]. A combination of antibiotic therapy and allopurinol successfully eradicated experimental pyelonephritis in the primate while protecting the renal tubular cells from reperfusion damage and preserving renal function [10].

This study demonstrates a significant release of extracellular elastase from PMN exposed to bacterial strains associated with renal damage. Further studies are needed to test for a causal relationship between extracellular release of elastase and the pathogenesis of renal damage in pyelonephritis. Elastase activity in the urine and plasma of patients with acute pyelonephritis need to be studied. Since an imbalance between elastase and α_1-proteinase inhibitor may exist in the renal parenchyma during the acute phase of infection, modulation of renal cortical damage with elastase inhibitors may be studied in vivo in animal models of pyelonephritis. Inhibitors of granulocyte elastase may be useful in modulating the course of pyelonephritis.

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References

1. Roitt IM (1984) Essential immunology, 5th edn. Blackwell, Boston, pp 182–185
2. Ormrod D, Cawley S, Miller T (1986) Neutrophil-mediated tissue destruction in experimental pyelonephritis. In: Kass EH, Svanborg EC (eds) Host-parasite interactions in urinary tract infections. University of Chicago Press, Chicago, pp 365–368
3. Glauser MP, Meylan P, Bille J (1987) The inflammatory response and tissue damage: the example of renal scars following acute renal infection. Pediatr Nephrol 1: 615
4. Skiles JW, Miao C, Sorcek R, Jacober S, Mui PW, Chow G, Weldon SM, Possanza G, Skoog M, Keirns J, Letts G, Rosenthal AS (1992) Inhibition of human leukocyte elastase by *N*-substituted peptides containing α,α-difluorostatone residues at P_1. J Med Chem 35: 4795
5. Blondin J, Janoff A (1976) The role of lysosomal elastase in the digestion of *Escherichia coli* proteins by human polymorphonuclear leukocytes. J Clin Invest 58: 971
6. Tanaka H, Shimazu T, Sugimoto H, Yoshioka T, Sugimoto T (1990) A sensitive and specific assay for granulocyte elastase in inflammatory tissue fluid using L-pyroglutamyl-L-prolyl-L-valine-*p*-nitroanilide. Clin Chim Acta 187: 173
7. Bergenfeld M, Axelsson L, Ohlsson K (1992) Release of neutrophil proteinase 4(3) and leukocyte elastase during phagocytosis and their interaction with proteinase inhibitors. Scand J Clin Lab Invest 52: 823
8. Carrell RW (1986) α_1-Antitrypsin: molecular pathology, leukocytes, and tissue damage. J Clin Invest 78: 1427
9. Roberts JA, Kaack MB, Baskin G (1990) Treatment of experimental pyelonephritis in the monkey. J Urol 143: 150
10. Dooley DC, Simpson JF, Meryman HT (1982) Isolation of large numbers of fully viable human neutrophils: a preparative technique using Percoll density gradient centrifugation. Exp Hematol 10: 591
11. Roberts JA, Kaack MB, Fussell EN (1989) Bacterial adherence in urinary tract infections: preliminary studies in a primate model. Infection 17: 401
12. Tanaka H, Ogura H, Yokota J, Sugimoto H, Yoshioka T, Sugimoto T (1991) Acceleration of superoxide production form leukocytes in trauma patients. Ann Surg 214: 187
13. Roberts JA (1991) Etiology and pathophysiology of pyelonephritis. Am J Kidney Dis 17: 1
14. Roberts JA, Roth JK Jr, Domingue G, Lewis RW, Kaack B, Baskin G (1992) Immunology of pyelonephritis in the primate model. V. Effect of superoxide dismutase. J Urol 128: 1394
15. Roberts JA, Kaack MB, Fussell EN, Baskin G (1986) Immunology of pyelonephritis. VII. Effect of allopurinol. J Urol 136: 960
16. Roberts JA (1986) In: Kass EH, Svanborg EC (eds) Host-parasite interactions in urinary tract infections. University of Chicago Press, Chicago, pp 369–375
17. Mundi H, Bjorksten B, Svanborg C, Ohman L, Dahlgren C (1991) Extracellular release of reactive oxygen species from human neutrophils upon interaction with *Escherichia coli* strains causing renal scarring. Infect Immun 59: 4168
18. Carp H, Janoff A (1979) In vitro suppression of serum elastase-inhibitory capacity by reactive oxygen species generated by phagocytosing polymorphonuclear leukocytes. J Clin Invest 63: 793
19. Ossana PJ, Test ST, Matheson NR, Regiani S, Weiss SJ (1986) Oxidative regulation of neutrophil elastase-alpha-1-proteinase inhibitor interactions. J Clin Invest 77: 1939
20. Desrochers PE, Mookhtiar K, Wart HE van, Hasty KA, Weiss SJ (1992) Proteolytic inactivation of α_1-proteinase inhibitor and α_1-antichymotrypsin by oxidatively activated human neutrophil metalloproteinases. J Biol Chem 267: 5005
21. Topley N, Steadman R, Mackenzie R, Knowlden JM, Williams JD (1989) Type 1 fimbriate strains of *Escherichia coli* initiate renal parenchymal scarring. Kidney Int 36: 609
22. Maillard JL, Favreau C, Vergely I, Reboud-Ravaux M, Joyeau R, Kobaiter R, Wakselman M (1992) Protection of vascular basement membrane and microcirulation from elastase-induced damage with a fluorinated b-lactam derivative. Clin Chim Acta 213: 75
23. Speer CP, Ruess D, Harms K, Herting E, Gefeller O (1993) Neutrophil elastase and acute pulmonary damage in neonates with severe respiratory distress syndrome. Pediatrics 91: 794