Correlation between Disease Stage and Activity of Elastase of PMN Origin in the Serum from IgA Nephropathy Patients

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Summary Activities of various enzymes including elastase were determined in sera from healthy control and from patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), IgA nephropathy (IgA NP), and other renal failures. In IgA NP, the activity of elastase increased significantly in parallel with the disease stage. It correlated well with the increase in the amount of polymorphonuclear leukocyte-elastase (PMN-elastase)• α_1 -antitrypsin complex assayed by ELISA, with the increase in myeloperoxidase (MPO) activity, and also with the increase in proteinuria. Studies on both substrate and inhibitor specificities of the elastase showed that the enzyme is indeed from PMN.

Key Words: IgA nephropathy, PMN-elastase, myeloperoxidase, serum, polymorphonuclear leukocyte

Polymorphonuclear leukocytes (PMN) are known to play a central role in the whole line of defense mechanism including antigen-antibody reactions and inflammation processes. When an invasion takes, which includes place in a certain location, PMN are attracted to the sites and release various kinds of substances, that cause a variety of local reactions at the sites. In some cases damage of tissues is known to be caused through lysosomal enzymes such as elastase released from PMN; adult respiratory distress syndrome (ARDS), inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and so on

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are included in this category [1-4]. In the case of Wegener's granulomatosis (WG), rapidly progressive glomerulonephritis (RPGN), and the Kawasaki's disease, participation of autoantibodies against PMN-derived myeloperoxidase (MPO) and elastase originated from PMN is well established [5-10]. Although the disease process of IgA nephropathy (IgA NP) has not been well elucidated yet, it is reasonable to assume that PMN play an important role also in this case.

In the present investigation, by assaying PMN-derived elastase activity in serum from IgA NP patients, we found that PMN are indeed deeply involved also in the disease process of IgA NP. Furthermore, the activities of several enzymes produced from PMN were compared among the sera from the patients with SLE, RA, chronic renal failure (CRF), IgA NP, and RPGN.

MATERIALS AND METHODS

Substrates and enzymes for enzyme assay. The sources of substrates and enzymes were as follows (see Table 1 for abbreviations): Lys-Ala•NA, Gly-Pro•NA from Bachem Feinchemikalein AG, Budendorf, Switzerland; PyrGlu-Pro-Val•pNA from Calbiochem Novabiochem AG, Lufelfingen, Switzerland; SucAla-Ala-Ala•pNA, PP-Glr, TMBZ, H₂O₂ and PMN-elastase from Wako Pure Chem. Ind., Osaka; NP-Man, NP-GlcNAc, PNA and porcine pancreatic elastase (P-elastase) from Sigma Chemical Co., St. Louis, MO; and PNP from Daiichi Chemicals Co., Tokyo.

Serum of patients. Control sera were obtained from 10 cases of healthy volunteers; and patients' sera, from 10 cases of SLE, 10 cases of RA, 12 cases of CRF, 7 cases of IgA NP and 15 cases of RPGN (Table 2). For additional

Table 1. List of the enzymes measured and their substrates.

Enzyme	Abbreviation	Substrate	References
Dipeptidylpeptidase II (EC 3.4.14.2)	DPP-II	Lys-Ala•NA	11
Dipeptidylpeptidase IV (EC 3.4.14.5)	DPP-IV	Gly-Pro•NA	12
Leukocyte elastase (EC 3.4.21.37)	PMN-elastase	PyrGlu-Pro-Val•pNA	13
Pancreatic elastase (EC 3.4.21.36)	P-elastase	SucAla-Ala-Ala•pNA	14
α -D-Mannosidase (EC 3.2.1.24)	Man-ase	NP-Man	15
N-Ac- β -D-glucosaminidase (EC 3.2.1.30)	GlcNAc-ase	NP-GlcNAc	15
β -Glucuronidase (EC 3.2.1.31)	β -Glr-ase	PP-Glr	16
Triacylglycerol lipase (EC 3.1.1.3)	Lipase	PNA	17
Alkaline phosphatase (EC 3.1.3.1)	Alk-phosph	PNP	18
Myeloperoxidase (EC 1.11.1.7)	MPO	H ₂ O ₂ /TMBZ	19

Abbreviations used: Lys-Ala•NA, L-lysyl-L-alanine β -naphthylamide; Gly-Pro•NA, glycyl-L-proline β -naphthylamide; PyrGlu-Pro-Val•pNA, L-pyroglutamyl-L-prolyl-L-valine *p*-nitroanilide; SucAla-Ala-Ala•pNA, succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide; NP-Man, *p*-nitrophenyl- α -D-mannopyranoside; NP-GlcNAc, *p*-nitrophenyl-N-acetyl- β -D-glucosaminide; PP-Glr, phenolphthalein- β -D-glucuronide; PNA, *p*-nitrophenylacetate; PNP, *p*-nitrophenylphosphate; H₂O₂/TMBZ, H₂O₂/3,3',5,5'-tetramethylbenzidine.

Table 2. Enzymatic changes in serum of patients with renal failure.

Enzyme	Specific activity $\pm$ SEM (nmol/min/ml)						
	Control (n=10)	SLE (n=10)	RA (n=10)	CRF (n=12)	IgA NP (n=7)	RPGN (n=15)	
DPP-II	4.17 $\pm$ 0.34	3.95 $\pm$ 0.40	3.35 $\pm$ 0.40	2.98 $\pm$ 0.23	2.83 $\pm$ 0.32	2.78 $\pm$ 0.30*	
DPP-IV	63.01 $\pm$ 1.68	$\downarrow$ 44.36 $\pm$ 3.75***	$\downarrow$ 45.86 $\pm$ 2.04***	$\downarrow$ 45.49 $\pm$ 2.00***	$\downarrow$ 46.48 $\pm$ 3.53***	$\downarrow$ 41.52 $\pm$ 1.56***	
Elastase ^a	5.67 $\pm$ 0.37	7.59 $\pm$ 1.31	8.21 $\pm$ 1.45	6.71 $\pm$ 0.62	$\uparrow$ 25.08 $\pm$ 7.42***	9.18 $\pm$ 1.77	
Man-ase	1.12 $\pm$ 0.05	1.69 $\pm$ 0.37	1.58 $\pm$ 0.32	2.05 $\pm$ 0.25	0.97 $\pm$ 0.30	1.91 $\pm$ 0.24	
GlcNAc-ase	8.46 $\pm$ 0.90	$\uparrow$ 16.15 $\pm$ 2.26***	12.01 $\pm$ 0.85	11.69 $\pm$ 0.91	11.01 $\pm$ 1.05	11.20 $\pm$ 0.67	
β -Glr-ase ^a	6.86 $\pm$ 0.73	12.89 $\pm$ 4.42	10.84 $\pm$ 0.91	6.50 $\pm$ 0.95	10.07 $\pm$ 1.46	9.17 $\pm$ 1.16	
Lipase	323.61 $\pm$ 10.69	$\downarrow$ 263.30 $\pm$ 10.35**	$\downarrow$ 206.46 $\pm$ 5.31***	$\downarrow$ 243.10 $\pm$ 9.58***	$\downarrow$ 273.63 $\pm$ 19.91*	$\downarrow$ 223.21 $\pm$ 13.32***	
Alk-phosphatase	6.74 $\pm$ 0.52	7.16 $\pm$ 0.58	6.90 $\pm$ 0.83	7.52 $\pm$ 0.96	5.86 $\pm$ 1.23	8.45 $\pm$ 1.43	
MPO ^b	1.43 $\pm$ 0.39	1.33 $\pm$ 0.42	1.32 $\pm$ 0.17	0.35 $\pm$ 0.14	3.06 $\pm$ 1.31*	0.61 $\pm$ 0.12	

^aElastase and β -Glr-ase activities were expressed as nmol of reaction products generated during 1 h of incubation per 1 ml of serum. ^bMPO activity was expressed as the increase in absorbance of oxidized products generated during 1 min of incubation per 1 ml of serum. SEM: standard error of mean. Significantly different from each control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; according to Bonferroni's t -test.

Table 3. Enzymatic changes in sera from patients with IgA NP.

Enzyme	Specific activity $\pm$ SEM	
	Control (n=25)	IgA NP (n=42)
Elastase (nmol/h/ml)	5.59 $\pm$ 0.39	11.60 $\pm$ 2.00*
MPO (Δ OD/min/ml)	1.25 $\pm$ 0.18	3.26 $\pm$ 0.49**

Significantly different from each control: * $p < 0.05$, ** $p < 0.01$; according to Student's t -test.

Control: age, mean $\pm$ SD = 22.6 $\pm$ 2.2 (male : female = 1 : 1). IgA NP: age, mean $\pm$ SD = 31.1 $\pm$ 14.3 (male : female = 2 : 5).

investigation, sera were also obtained from 25 cases of healthy volunteers and 42 cases of IgA NP patients (Table 3).

Determination of enzyme activities. The serum was dispensed into micro-well plates (Nuncclon, F96, Roskilde, Denmark) for determination of aminopeptidases, endopeptidases, glycosidase, lipase, alkaline phosphatase, and myeloperoxidase activities. The respective substrates were then added, followed by incubation at 37°C. For the dipeptidylpeptidase (DPP) assays, 50 μ l of 4 mM β -naphthylamide derivatives of peptides was used as the substrate, and the reaction was stopped by addition of 100 μ l of 6 mM Fast Garnet GBC. The absorbance at 525 nm was determined by a microplate reader 3550 (Bio-Rad, Richmond, VA) [11, 12]. For the PMN-elastase and P-elastase assays, 25 μ l of 2.5 mM PyrGlu-Pro-Val•pNA and of SucAla-Ala-Ala•pNA respectively, were used. After an incubation for 16 h at 37°C, the absorbance at 405 nm was determined with a microplate reader [13, 14].

For the Man-ase and GlcNAc-ase assays, 30 μ l of 20 mM *p*-nitrophenyl derivatives was used as the substrate, and the reaction was stopped by addition of 100 μ l of 0.6 M glycine/NaOH (pH 12.7) after an incubation for 60 min at 37°C. The absorbance at 405 nm after stand for 15 min at room-temperature was determined with a microplate reader [15]. For the β -Glr-ase assay, 25 μ l of 5 mM PP-Glr was incubated for 16 h at 37°C with serum, and the reaction was stopped by addition of 100 μ l of 0.17 M glycine/NaOH (pH 12.7). The absorbance at 550 nm after stand for 15 min at room-temperature was determined with a microplate reader [16]. For the lipase assay, 25 μ l of 4 mM PNA was used and the absorbance at 405 nm was determined with a microplate reader [17] following an incubation for 60 min at 37°C. For the Alk-phosph assay, 10 μ l of 0.1 M PNP was used, and the absorbance at 405 nm was determined with a microplate reader [18] following an incubation for 45 min at 37°C. For the MPO assay, 180 μ l of substrate-buffer mixture consisted of 1.7 mM TMBZ and 0.38 mM H₂O₂ was used in a 30-min incubation at 37°C, and the absorbance at 655 nm was determined [19]. The references for the assay methods and substrates used are listed in Table 1 [11–19]. All the enzyme assays in serum were done in the range covering linearity of the time-concentration relationship. The assays were done in triplicate, and their standard deviations were within 10% of the average values. For the assays, the units of enzyme activities were expressed as nmol of reaction products generated during 1 min of incubation per 1 ml serum.

Effect of elastatinal on serum elastase activity. Fifty microliters of 2 mM PyrGlu-Pro-Val•pNA or SucAla-Ala-Ala•pNA as substrate was mixed with 50 μ l of PMN-elastase or P-elastase and elastatinal (1 μ g/ml) [20] and incubated at 37°C. The absorbance at 405 nm was then determined with a microplate reader.

Determination of PMN-elastase levels. PMN-elastase levels were determined as PMN-elastase• α_1 -antitrypsin complex by use of PMN ELASTASE (Merck Immunoassay, Merck, Darmstadt).

RESULTS AND DISCUSSION

Enzymatic activities in control and sera from patients with SLE, RA, CRF, IgA NP, and RPGN are shown in Table 2. The activities of DPP-IV and lipase were significantly decreased in all diseases. The ratio of DPP-II to DPP-IV was increased in SLE and RA, but not in CRF, IgA NP, and RPGN. We think that the increase in this ratio is characteristic of collagen disease [21] but has no relation to renal failure. A significant increase in GlcNAc-ase was noticed in the case of SLE. On the other hand, both elastase and MPO activities in IgA NP patients were significantly high (Table 2).

To confirm the increased activities of both elastase and MPO in IgA NP, we used additional sera from IgA NP patients. Indeed, these activities were increased significantly in those patients (Table 3). Furthermore, the relationship between exacerbation of the disease, with stages based on Sakaguchi's classification [7], and the enzyme activities was examined. As shown in Fig. 1, the activities of elastase and MPO increased significantly along with the progression of the disease

Fig. 1. Distribution of enzyme activities on disease stages of IgA NP. Open circles represent the mean $\pm$ SEM. Significantly different from control, * $p < 0.05$, ** $p < 0.01$, by Bonferroni's t -test. II, III, IV represent histopathological form on Sakaguchi's classification.

stages. Figure 2 shows that proteinuria also increased significantly corresponding with the disease stage and that its increase correlated well with the increases in elastase and MPO.

PMN-elastase hydrolyzed PyrGlu-Pro-Val-pNA as a substrate but it does not SucAla-Ala-Ala-pNA. On the other hand, P-elastase hydrolyzed SucAla-Ala-Ala-pNA, but not PyrGlu-Pro-Val-pNA. Furthermore, elastatinal, a specific inhibitor of P-elastase, only weakly inhibited PMN-elastase at all in control or IgA NP samples assayed with PyrGlu-Pro-Val-pNA as a substrate. Our result based on the specificity of both substrates and the inhibitor clearly show that the elastase activity found in the serum from IgA NP patients indeed originated from PMN.

Ninety percent of PMN-elastase in the serum is in an inactive form complexed with α_1 -antitrypsin, but the remaining 10% is bound to α_2 -macroglobulin retaining an ability to disintegrate low-molecular substrates [22]. It is reasonable to assume that the elastase activity detected in the serum is due to a bound form complexed with α_2 -macroglobulin. The amount of PMN-elastase- α_1 -antitrypsin complex was determined by the ELISA method. Figure 3 shows that the amount of the complex increased significantly along with the disease stage: 3.6 fold over the control in the stage II, 4 fold in the stage III, and 6 fold in the stage IV. Furthermore, a highly positive correlation ($r=0.701$) was demonstrated between the activity of PMN-elastase and the amount of PMN-elastase- α_1 -antitrypsin complex (Fig. 4).

IgA NP occurs in a large population of chronic glomerulonephritis patients, and about 40% of IgA NP patients will become into end-stage of renal failure [23]. In the disease process of IgA NP, our presumption is that PMN become activated at the inflammation sites and release various enzymes with potent tissue-damaging

Fig. 2. Proteinuria on disease stages of IgA NP. II, III, and IV represent histopathological form on Sakaguchi's classification. * $p < 0.05$, ** $p < 0.01$.

Fig. 3. PMN-elastase levels in serum of patients with IgA nephropathy. PMN-elastase • α_1 -antitrypsin complex was determined by ELISA. Each value represents the mean $\pm$ SEM. Significantly different from the control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; according to Bonferroni's t -test.

Fig. 4. Correlation between PMN-elastase • α_1 -antitrypsin complex and elastase activities in IgA NP patients.

properties such as elastase or MPO, a result being progression of the disease. Our results suggest that determination of the activity of elastase in the serum by use of PyrGlu-Pro-Val-pNA as a substrate or determination of the amount of PMN-elastase • α_1 -antitrypsin complex will be a useful means of monitoring the procession of IgA NP.

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