BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **244,** 45–49 (1998)
ARTICLE NO. RC988203

Increased Pentosidine, an Advanced Glycation End Product, in Plasma and Synovial Fluid from Patients with Rheumatoid Arthritis and Its Relation with Inflammatory Markers

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Received January 23, 1998

Pentosidine is an advanced glycation end product (AGE) formed by combined processes of glycation and oxidation (glycoxidation) between carbohydrate-derived carbonyl group and protein amino group. Recent studies demonstrated the increased pentosidine levels not only in diabetic patients with hyperglycemia but also in normoglycemic uremic patients due to increased oxidative stress. Rheumatoid arthritis (RA) is a state of increased oxidative stress associated with chronic inflammation. This suggested an enhanced glycoxidation reaction and increased AGE levels in RA patients. In the present study, we therefore determined, by high performance liquid chromatographic (HPLC) assay, the concentrations of pentosidine in plasma and synovial fluid from 22 patients with rheumatoid arthritis (RA) and compared their levels with those in 17 patients with osteoarthritis (OA), 26 diabetic patients, and 25 normal subjects. The levels of inflammatory markers and markers of tissue destruction, metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), were also measured in the same samples. Pentosidine levels in plasma and synovial fluid from RA patients were significantly higher than those in OA patients, diabetic patients, and normal subjects. There was a significant correlation between the pentosidine levels in plasma and those in synovial fluid. Among markers of inflammation and matrix destruction, pentosidine levels in plasma from RA patients were correlated with the levels of C-reactive protein (CRP), erythrocyte sedimentation rate, white blood cell count, and platelet count. Multiple stepwise regression analysis reveals an independent influence of CRP on plasma pentosidine levels. In conclusion, pentosidine levels are significantly higher in plasma and synovial fluid from RA patients and may be useful as a biomarker of chronic inflammation in RA patients. © 1998 Academic Press

Abbreviations used: AGEs, advanced glycation end products; CRP, C reactive protein; ELISA, enzyme-linked immunosolvent assay; HPLC, high performance liquid chromatography; MMP, matrix metalloproteinase; OA, osteoarthritis; RA, rheumatoid arthritis; TIMP, tissue inhibitor of metalloproteinase.

Advanced glycation end products (AGEs) are formed, over months, by non-enzymatic glycation and oxidation ("glycoxidation") reactions between carbohydrate-derived carbonyl group and protein amino group, known as the Maillard reaction (1). Recent studies have demonstrated the increased levels of pentosidine (2), a well characterized AGE structure, in plasma proteins and skin collagen of diabetic patients (3). This rise has been ascribed to hyperglycemia: the levels of pentosidine both in the plasma and in a variety of tissues are correlated with the levels of fructoselysine, a direct result of hyperglycemia (3). The pentosidine levels in skin correlate well with the severity of diabetic complications (4). Also, in hemodialysis patients with end-stage renal failure, a dramatic increase in pentosidine was demonstrated in plasma proteins and skin collagen, irrespective of the presence or absence of diabetes (5). Pentosidine thereby has been implicated in long-term complications associated with uremia (6). This rise has been ascribed to increased oxidative stress of uremia: indeed, the plasma pentosidine levels are correlated with the oxidative stress marker levels in uremia (7).

AGE modified proteins are thought to contribute to normal tissue remodeling. However, under pathological conditions such as hyperglycemia in diabetes or increased oxidative stress in uremia, the accumulation of AGE-modified proteins might lead to tissue damage through a variety of mechanisms: an alteration of the structure and function of tissue proteins (1) and the

0006-291X/98 $25.00

stimulation of cellular responses such as monocyte chemotaxis, macrophage secretion of inflammatory cytokines, synovial cell production of collagenase, and osteoclast-induced bone resorption (8, 9). AGEs thus have been implicated in the pathogenesis of diabetic and uremic complications including atherosclerosis, diabetic nephropathy, and bone and joint destruction associated with dialysis-related amyloidosis (1, 6, 10).

Rheumatoid arthritis (RA) might be a state of increased oxidative stress due to chronic inflammation, as suggested by increased lipid peroxidation (11), decreased glutathione level (12), increased oxidative DNA damage assessed by 8-oxo-7-hydrodeoxyguanosine (13), decreased sulphydryl (SH) level (14), and increased mitochondrial radical production (15). These findings suggest an enhanced glycoxidation reaction and increased AGE levels in RA patients, especially in the affected joints, and led us to measure pentosidine levels in plasma and synovial fluid from RA patients.

In the present study, we demonstrate for the first time that pentosidine levels in plasma and synovial fluid from RA patients were significantly higher, as compared to those from osteoarthritis (OA) patients, diabetic patients, and normal subjects. We also demonstrate that pentosidine levels in plasma from RA patients were correlated with the levels of markers of inflammation. Pentosidine levels might thus be taken as a biomarker of chronic inflammation.

MATERIALS AND METHODS

Patients and samples. We previously demonstrated that plasma pentosidine level is significantly influenced by the quality of glycemic control and renal function (5). In order to avoid the influence of overt hyperglycemia and renal failure, we selected subjects with normoglycemia defined as a plasma fructoselysine level below 250 nmol/ml and hemoglobin A1c level below 5.0%, and normal renal function defined as a plasma creatinine level below 1.0 mg/dl and no proteinuria by Albustix (Bayer-Sankyo, Tokyo, Japan).

Twenty two patients with RA, regularly followed at the clinic of Department of Orthopedic Surgery, Nagoya University School of Medicine, were included after obtaining an informed consent. Complete histories and physical examination were obtained in each patient. Every patient fulfilled the diagnostic criteria for RA of the American College of Rheumatology. All patients were being treated with nonsteroidal antiinflammatory drugs (NSAID). In addition, some patients were receiving other antirheumatic drugs including gold sodium thiomalate (13), D-penicililamine (4), bucillamine (3), and prednisolone (11).

Synoval fluid was asprirated from the knee joints of RA patients as a part of their therapeutic regimen. Blood samples were also collected at the time of synovial fluid aspiration and measured for levels of glucose, fructoselysine, creatinine, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), white blood cell count, and platelet count by routine methods at the Central laboratory of Nagoya University Hospital.

Blood samples were also collected and analyzed, with informed consent, in 17 OA patients, 26 non-insulin dependent diabetes mellitus, 25 healthy non-diabetic subjects without any joint destruction. Synoval fluid was obtained from the knee joints of OA patients.

Pentosidine measurement by HPLC assay. The plasma and synovial fluid samples were lyophilized, hydrolyzed by 100 μl of 6 N HCl for 16 hours at 110 °C under nitrogen, followed by neutralization with 100 μl of 5 N NaOH and 200 μl of 0.5 M phosphate buffer (pH 7.4), then filtered through a 0.5 μm-pore filter, and diluted with phosphate buffered saline. The pentosidine content was analyzed on a reverse-phase HPLC according to our previous methods (5, 10). Briefly, a 50 μl solution of acid hydrolysate of plasma was injected into an HPLC system and separated on a C18 reverse-phase column (Waters, Tokyo, Japan). The effluent was monitored using a fluorescence detector (RF-10A: Shimadzu, Kyoto, Japan) and an excitation-emission wavelength of 335/385 nm. Synthetic pentosidine was used as a standard. The substance in the specimens, detected at the same retention time as authentic pentosidine, was confirmed as pentosidine by fast atom bombardment-mass spectrometry. Detection limit was 0.1 pmol of pentosidine *per* mg of plasma proteins.

ELISAs for MMPs and TIMPs. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) were evaluated for assessing joint cartilage destruction. MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and TIMP-1 and TIMP-2 levels in plasma and synovial fluid were determined by ELISAs as described previously (16).

Statistical analysis. All data were expressed as mean ± SD. Unpaired *t*-test or one way analysis of variance (ANOVA) were used for statistical evaluation. Correlation was assessed by linear regression analysis. Multiple stepwise linear regression was used to determine the factor(s) that influence plasma pentosidine levels. A P value less than 0.05 was considered statistically significant.

RESULTS

Increased pentosidine levels in plasma and synovial fluid of RA patients. Pentosidine levels in acid hydrolysates of synovial fluid from RA patients with normoglycemia and normal renal function, were significantly higher ($P < 0.01$) than those from OA patients (161.4 ± 114.8 vs. 84.3 ± 26.9 pmol/ml: closed columns in Fig. 1). There were no significant differences in renal function assessed by serum creatinine, glycemic control assessed by fructoselysine levels, patient age, and total plasma protein levels between RA and OA patients (Table 1). The pentosidine levels expressed *per* mg of protein were higher ($P < 0.01$) in synovial fluid from RA patients than in the plasma (11.02 ± 8.53 vs. 4.01 ± 3.27 pmol/mg of protein).

Interestingly, pentosidine levels in synovial fluid from RA patients were correlated with their plasma pentosidine levels ($r^2 = 0.648$, $P < 0.001$) (Fig. 2). This correlation between the levels of pentosidine in plasma and that in synovial fluid was also observed in OA patients ($r^2 = 0.604$, $P < 0.01$).

Plasma pentosidine levels in RA patients (234.4 ± 168.2 pmol/ml) were significantly higher than in OA patients (128.1 ± 40.0 pmol/ml, $P < 0.01$), diabetic patients (135.8 ± 45.9 pmol/ml, $P < 0.01$), and normal subjects (119.6 ± 23.9 pmol/ml, $P < 0.001$) (open columns in Fig. 1). There were no significant differences in serum creatinine, patient age, fructoselysine (except for diabetic patients), and total plasma protein levels between RA patients and the other three control groups (Table 1). There was no significant difference in plasma pentosidine levels between the RA patients treated

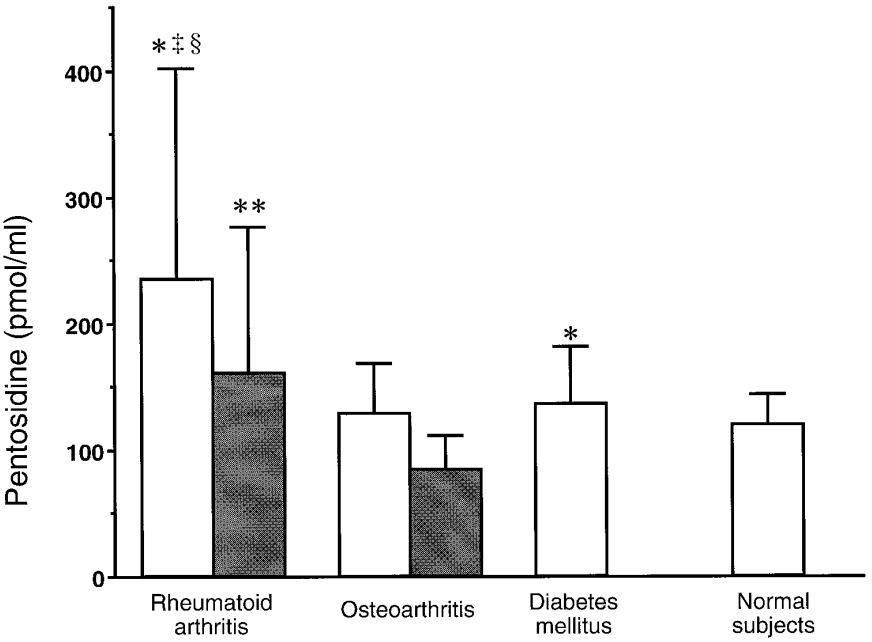

FIG. 1. Plasma pentosidine levels in RA patients (n = 22), OA patients (n = 17), diabetic patients (n = 26), and healthy normal subjects (n = 25). The pentosidine level in acid hydrolysate of plasma was determined by HPLC assay. Data are expressed as means ± SD. $^{**}P < 0.01$ vs. synovial fluid from OA patients, $^{*}P < 0.001$ vs. normal subjects, $\ddagger P < 0.01$ vs. diabetic subjects, and $\S P < 0.01$ vs. OA patients. Closed column, synovial fluid; open column, plasma.

with and without steroid (268.0 ± 194.9 and 200.7 ± 137.5 pmol/ml, respectively). The levels of pentosidine in the plasma samples from patients with acute respiratory infection (n = 11; serum creatinine, 0.75 ± 0.28 mg/dl; age, 70.0 ± 24.3 year; total protein, 7.18 ± 0.71 g/dl; CRP, 0.5–17.8 mg/dl) were 114.3 ± 48.3 pmol/ml (no significant difference versus normal subjects).

Relation of pentosidine level with markers of inflammation and matrix degradation. Plasma pentosidine levels in RA patients were clearly influenced by their inflammatory status (Table 2): plasma pentosidine levels were significantly correlated with acute phase reactants, such as CRP ($r^2 = 0.386$, $P < 0.01$), ESR ($r^2 = 0.201$, $P < 0.05$), white blood cell count ($r^2 = 0.242$, $P < 0.05$), and platelet count ($r^2 = 0.206$, $P < 0.05$).

Plasma pentosidine levels were correlated with markers of matrix degradation such as plasma MMP-3 ($r^2 = 0.120$) and TIMP-2 ($r^2 = 0.095$), but this correlation did not reach statistical significance. Plasma pentosidine levels did not correlate with the levels of MMP-1, 2, 8, and 9 and TIMP-1 in plasma and synivial fluid (Table 2).

Multiple stepwise regression analyses revealed an independent influence of CRP ($r^2 = 0.454$, $P < 0.01$) on plasma pentosidine levels, among regressors including serum creatinine, fructoselysine, patient age, total protein, CRP, ESR, white blood count, platelet count, MMPs, and TIMPs.

DISCUSSION

The pentosidine levels in synovial fluid in RA patients were significantly higher than those in OA patients. The pentosidine levels were also higher in RA plasma than in OA plasma and, interestingly, than in diabetic plasma with hyperglycemia. The mechanism of increased pentosidine in plasma and synovial fluid from RA patients remains unclear. AGE accumulation including pentosidine is enhanced in diabetes as a result of sustained hyperglycemia. In RA patients, by contrast, despite an even more striking rise in the plasma pentosidine levels, glucose levels are normal. We, therefore, postulated the existence in RA of factor(s) catalyzing the formation of pentosidine.

Pentosidine formation is closely linked not only to glycation but also to oxidative processes, hence its qualification as a "glycoxidation" product. Wolff *et al.* have indeed demonstrated that reducing sugars can autoxidize by metal-catalyzed oxidative processes and generate H_2O_2, reactive oxygen intermediates, and ketoaldehydes, which contribute to chromo and fluorophobic alterations of proteins now taken as characteristic of AGEs (17). Baynes and his colleagues have also shown that oxidation is an essential process in the formation of pentosidine (18). That pentosidine is the product of combined processes of glycation and oxidation is further supported by *in vitro* evidence that pentosidine production is prevented when oxygen is absent from the incubation medium (18).

Several lines of evidence demonstrate that RA is associated with an increased oxidative stress due to chronic inflammation (11–15). It appears, therefore, likely that the RA associated oxidative stress contributes to the generation of AGEs including pentosidine.

TABLE 1

Profile of Subjects Examined in This Study

		Rheumatoid arthritis (RA)	Osteoarthritis (OA)	Diabetes mellitus (DM)	Normal subjects (NS)
Serum creatinine	(mg/dl)	0.79 ± 0.20	0.83 ± 0.21	0.68 ± 0.18	0.70 ± 0.12
Patient age	(year)	68.7 ± 9.1	67.1 ± 10.2	62.0 ± 11.6	61.0 ± 8.1
Fructoselysine	(μmol/l)	216.2 ± 25.0	231.8 ± 22.1	537.9 ± 210.8*	252.8 ± 31.4
Total protein	(g/dl)	7.02 ± 0.45	6.91 ± 0.41	7.19 ± 0.56	7.26 ± 0.39
Pentosidine	(pmol/ml)	234.4 ± 168.2‡§	128.1 ± 40.0	135.8 ± 45.9	119.6 ± 23.9

Note. Data are expressed as means ± SD.
* $P < 0.0001$ versus RA, OA and NS.
‡ $P < 0.01$ versus OA and DM.
§ $P < 0.001$ versus NS.

The oxidative stress theory in enhanced AGE generation is also suggested in uremia and supported by the recent demonstration in uremic plasma of a correlation between the levels of pentosidine and oxidative stress markers (7). A higher pentosidine levels in synovial fluid (expressed *per* mg of protein), as compared to plasma, supports the notion that the oxidative stress in RA increased pentosidine generation, because the primary tissue affected in RA is the joint structure.

Plasma pentosidine levels were correlated with inflammatory markers such as CRP, ESR, white blood count, and platelet count. These biochemical variables are acute phase reactants rather than chronic inflammatory markers. By contrast, plasma pentosidine represents much more long-term oxidative stress: in the plasma, pentosidine exists exclusively in the albumin fraction (5) and, therefore, the plasma pentosidine levels depend on the albumin turnover. Thus, plasma pentosidine, by contrast with acute phase reactants, may be more suitable as a marker of chronic inflammation. The different degree of disease activity observed in RA patients might explain for the wide range of standard deviation for pentosidine levels.

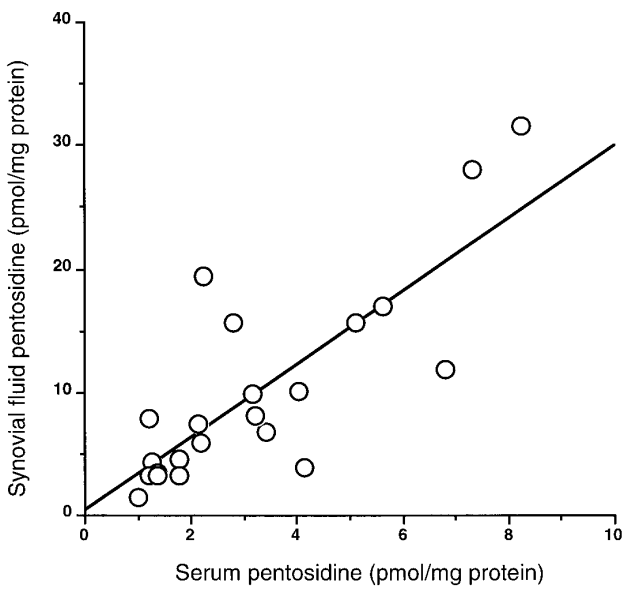

FIG. 2. Correlation between the pentosidine levels in plasma and in synovial fluid from RA patients. Plasma pentosidine levels correlate with those in synovial fluid in RA patients (n = 22, $P < 0.001$, $r^2 = 0.648$). The equation of the line is $y = 2.972x + 0.415$.

An increase of MMP activity in relation to TIMP activity has been revealed at sites of cartilage destruction (16). Plasma pentosidine levels were correlated with MMP-3, which is the most abundant MMP in the synovial fluids (Table 2). MMP-3 is shown to be the most important degradative enzyme in the cartilage breakdown (19): indeed, it has been shown that plasma concentrations of MMP-3 correlates with CRP (16). Plasma pentosidine levels were also correlated with TIMP-2. It is of note that inflammatory cytokines are found, at high concentrations and in correlation with acute phase reactants, in the plasma and synovial fluids of RA patients, and that these inflammatory cytokines stimulate the production of MMPs and TIMPs (16, 20).

Interestingly, recent studies have demonstrated that AGEs are endowed with biological activitie which can partly account for bone and joint destruction through mechanism including macrophage secretion of inflammatory cytokines, synovial production of collagenase, and osteoclast-induced bone resorption (8, 9). The possible involvement of AGEs in bone and joint destruction associated with dialysis-related amyloidosis, a serious complication of chronic uremia, has been demonstrated (6, 10). However, at this stage, it remains unknown whether the increased AGE modified proteins in RA patients merely represent oxidative stress of chronic inflammation or actively participate in the bone and joint destruction of RA. Further studies will be necessary to address this issue.

In conclusion, pentosidine levels are significantly higher in plasma and synovial fluid from RA patients and might be taken as a biomarker of chronic inflammation. A long-term, prospective study will be able to demonstrate whether elevated plasma pentosidine is

TABLE 2

Relation of Pentosidine Level with Markers of Inflammation and Matrix Degradation

	Mean ± SD		Correlation (r^2)
Serum creatinine	0.79 ± 0.20	(mg/dl	0.170
Patients age	68.7 ± 9.1	(year)	0.003
Fluctoselysine	216.2 ± 25.0	(μmol/l)	0.007
C-reactive protein	4.59 ± 4.10	(μg/ml)	0.386*
White blood cell count	7600 ± 2230	(count/μl)	0.242†
Platelet count	297000 ± 78500	(count/μl)	0.206†
Erythrocyte sedimentation rate	46.0 ± 27.8	(mm/60 min.)	0.201†
RA hemagglution	297.0 ± 78.5	(times dilution)	0.033
Serum MMP-1	58.51 ± 44.29	(ng/ml)	0.059
Serum MMP-2	592.564 ± 143.297	(ng/ml)	0.058
Serum MMP-3	490.42 ± 700.00	(ng/ml)	0.120
Serum MMP-8	98.26 ± 74.95	(ng/ml)	0.036
Serum MMP-9	266.35 ± 133.82	(ng/ml)	0.000
Serum TIMP-1	314.91 ± 207.82	(ng/ml)	0.001
Serum TIMP-2	26.62 ± 4.85	(ng/ml)	0.095
Synovial fluid MMP-1	1308.23 ± 1154.35	(ng/ml)	0.003
Synovial fluid MMP-2	2063.7 ± 713.5	(ng/ml)	0.048
Synovial fluid MMP-3	11220.6 ± 8078.6	(ng/ml)	0.045
Synovial fluid MMP-8	4142.64 ± 3058.26	(ng/ml)	0.052
Synovial fluid MMP-9	2360.4 ± 1833.0	(ng/ml)	0.058
Synovial fluid TIMP-1	1068.0 ± 561.9	(ng/ml)	0.006
Synovial fluid TIMP-2	79.81 ± 60.21	(ng/ml)	0.000

Note. Data are expressed as means ± SD.
* $P < 0.01$.
† $P < 0.05$.

indeed a predictor of clinical complications in RA patients.

ACKNOWLEDGMENTS

We thank Professor Kusuki Nishioka for critically reviewing the manuscript and providing us valuable comments. This study was supported by a grant from Research for the Future Program of the Japan Society for the Promotion of Science (96L00303).

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