CLINICAL STUDY

Possible involvement of matrix metalloproteinase-3 in the pathogenesis of macroprolactinaemia in some patients with rheumatoid arthritis

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Abstract

Objective: Macroprolactin primarily comprises a complex of prolactin (PRL) and IgG molecules, particularly anti-PRL autoantibodies. However, it is unknown why autoantibodies against PRL develop in certain subjects. This study aimed to elucidate post-translational modifications in the PRL molecule that may be involved in the pathogenesis of macroprolactinaemia.

Methods: Macroprolactinaemia was screened with a polyethylene glycol method in 238 patients with rheumatoid arthritis (RA) and 302 control subjects and confirmed by gel chromatography. We examined the relationship between macroprolactinaemia and several RA-related laboratory tests including matrix metalloproteinase-3 (MMP-3) and anti-cyclic citrullinated peptide (CCP) antibody titres. The effect of MMP-3 on the PRL molecule was examined by western blotting.

Results: Patients with RA exhibited a significantly higher prevalence of macroprolactinaemia (15/238; 6.3%) than the young control subjects (5/219 subjects; 2.3%), but the prevalence was not different from that observed in the elderly control subjects (5/83 subjects; 6.0%). The prevalence of macroprolactinaemia in patients with elevated MMP-3 levels (9.68%) was significantly higher than that in those with normal MMP-3 levels (2.63%). Digestion of PRL with MMP-3 produced vasoinhibins with several molecular species. Serum total and free PRL levels in RA patients were higher than those in the age- and gender-matched control subjects. The levels of macroprolactin were not significantly correlated with those of RA-specific anti-CCP antibody.

Conclusions: We speculate that elevated MMP-3 levels may lead to the formation of new epitopes on the PRL molecule that might trigger an immune response to produce anti-PRL autoantibodies in some patients with RA. Such post-translational modifications may possibly contribute to the increased prevalence of macroprolactinaemia in elderly subjects.

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Introduction

Macroprolactinaemia is one of the major causes of hyperprolactinaemia (1, 2, 3, 4, 5). In patients with macroprolactinaemia, the predominant form of prolactin (PRL) in the serum is macroprolactin, with a molecular mass >150 kDa. The slow clearance of macroprolactin from the circulation, due to its large molecular size, is likely to cause hyperprolactinaemia (6). The prevalence of macroprolactinaemia has been reported to be 10–26% in patients with hyperprolactinaemia (1, 2, 3, 4) and 3.68% in the general population (5).

Macroprolactin is a heterogeneous molecule, but it mostly comprises PRL in a complex with IgG, in particular, with anti-PRL autoantibodies (4, 5, 6, 7, 8, 9).

However, it is not known why anti-PRL autoantibodies develop in certain subjects. In our previous study, we found that the prevalence of macroprolactinaemia was higher in aged subjects (5). Ageing is a progressive, degenerative process closely related to chronic inflammation; thus, with increasing age, more proteins are susceptible to post-translational modifications (10). Modifications in self-antigens that arise later in life may trigger reactions in the immune system. Autoantibodies against modified proteins may also cross-react with the original, unmodified proteins. We hypothesized that aberrant post-translational modifications in the PRL molecule could induce the production of anti-PRL autoantibodies.

Matrix metalloproteinase (MMP)-3 is an enzyme that degrades connective tissue matrix components,

including proteoglycan, laminin, fibronectin and collagen (11, 12). MMP-3 is synthesized by synovial fibroblasts and chondrocytes, and its production is highly enhanced in rheumatoid arthritis (RA), a common autoimmune disorder. MMP-3 has a broad spectrum of substrate specificities, and PRL is one of its substrates (13). MMP-3 breaks down the PRL molecule into several fragments, known as vasoinhibins. Vasoinhibins comprise a family of peptides that suppress angiogenesis and promote apoptosis-mediated vascular regression (14). This post-translational modification of the PRL molecule may be involved in the induction of the production of anti-PRL autoantibodies.

In this study, we examined the relationship between macroprolactinaemia and MMP-3 and other inflammatory markers in patients with RA to clarify the mechanism underlying the development of autoantibodies against PRL.

Subjects and methods

Subjects

Macroprolactinaemia was screened in 238 patients with RA (200 women and 38 men, aged 60.7 ± 12.5 years (mean $\pm$ s.d.)) who fulfilled the criteria for RA (15). We used 1987 ACR criteria for RA, not the 2010 ACR/EULAR classification, because most patients were diagnosed with RA before 2010. Serum samples were collected from 240 patients with RA, who gave informed consent to participate in the study from September 2011 to July 2012. Two patients were excluded from the study because they were taking domperidone, which could elevate serum PRL levels, for gastric symptoms. The disease duration of 238 patients with RA ranged from 0.3 to 44 years (13.9 ± 7.2 years). Their RA stages based upon the appearance of joints on the X-rays were as follows: 14 in stage I (no bone damage on X-rays), 16 in stage II (evidence of bone thinning around a joint), 112 in stage III (evidence of cartilage and bone damage), and 96 in stage IV (evidence of osteoporosis and ankylosis). The functional statuses of RA patients were as follows: 61 in class I (completely able to perform the usual activities of daily living), 118 in class II (limited performance in activities outside of work), 59 in class III (limited performance in work and other activities), and none in class IV (limited in the ability to perform usual self-care). These patients had received several combinations of the following drugs to suppress inflammation and immune reactions: biological agents such as infliximab ($n=93$), methotrexate ($n=145$), prednisolone ($n=107$) and tacrolimus ($n=37$); disease-modifying anti-rheumatic drugs such as salazosulphapyridine ($n=78$); and non-steroidal anti-inflammatory drugs such as diclofenac ($n=79$). Control serum samples were acquired from 83 age- and gender-matched elderly control subjects

(69 women and 14 men, aged 59.0 ± 2.4 years) and 219 gender-matched young control subjects (185 women and 34 men, aged 29.4 ± 2.6 years) who were working at Kobe City General Hospital. The control subjects agreed to allow collecting extra sera than that drawn for their medical check-up to be used for this study, with the condition that only gender and age would be available as clinical information. Therefore, the possibility could not be ruled out that some control subjects might have RA.

Blood was drawn in the morning, and serum was separated and stored at -80°C until use. This study was approved by the ethical committees of Kobe City General Hospital and Miyashima RA and Orthopaedic Hospital.

Polyethylene glycol precipitation and PRL assays

To determine free PRL concentrations, macroprolactin was removed from the serum samples (50 μl) by mixing vigorously with 50 μl cold polyethylene glycol (PEG; molecular weight 6000, 25% in water) and centrifuging at 9100 *g* for 10 min. The supernatant was diluted tenfold with phosphate buffer (0.01 M, pH 7.0) that contained 0.4 M NaCl and 0.1% BSA (final serum dilution, 20-fold). To determine total PRL concentrations, additional serum samples were treated identically, but with water instead of PEG. Free PRL was defined as the PRL concentration in the supernatant after PEG precipitation; total PRL was defined as the PRL concentration in the water-treated sample. The PEG-precipitable PRL (%), which represents the amount of macroprolactin, was calculated as follows: (total PRL – free PRL)/total PRL $\times$ 100. When the amount of macroprolactin precipitated with PEG was $>60\%$ (PRL recovery $<40\%$), macroprolactinaemia was diagnosed.

PRL concentrations were measured in duplicate with an enzyme immunoassay for human PRL as described previously (5). In brief, serum samples were incubated with polystyrene balls (Precision Plastic Ball Co., Chicago, IL, USA) that were coated with anti-human PRL antiserum (NIDDK-Anti-human PRL IC-5) at 37°C for 6 h. After washing, the balls were incubated at 4°C overnight with affinity-purified, anti-human PRL Fab' fragments conjugated to HRP. This was followed by incubation at 20°C for 6 h. After washing, the peroxidase activity bound to the balls was assayed in an enzyme reaction with 3-(*p*-hydroxyphenyl) propionic acid (Aldrich Chemical Co., Milwaukee, WI, USA) as the substrate. Results were evaluated by measuring fluorescence intensity with a spectrofluorophotometer (FP-6200ST; JASCO Co., Tokyo, Japan), with excitation at 320 nm and emission at 405 nm.

Highly purified recombinant human PRL (human PRL-RP-2) and anti-human PRL polyclonal (rabbit) antibodies were provided by Dr A F Parlow of the Harbour-UCLA Medical Centre, National Hormone

and Peptide Program, Torrance, CA, USA (parlow@humc.edu). The upper reference value of serum total PRL was 525 mIU/l (conversion factor = 1/35 for mIU/l to μ g/l). The limit of detection was 0.175 mIU/l. The intra- and inter-assay coefficients of variation were 4 and 5% respectively, based on a serum sample with a total PRL concentration of 178.5 mIU/l.

Gel filtration chromatography

Gel filtration chromatography was performed with a 1 $\times$ 60 cm column of Ultrogel AcA 44 (IBF Biotechnics, Villeneuve La Garenne, France), equilibrated with 0.01 M sodium phosphate buffer (pH 7.0), which contained 0.1 M NaCl, 0.1% BSA and 0.01% NaN₃. Serum samples (100 μ l) were applied to the column, and 1 ml fractions were collected for the determination of PRL concentrations. The column was calibrated with various molecular weight markers (Sigma).

Biochemical markers

Serum levels of MMP-3 were measured with Panaclear MMP-3 (Latex; Sekisui Medical Co., Tokyo, Japan). The reference intervals were 36.9–121.0 ng/ml for men and 17.3–59.7 ng/ml for women. Values greater than the maximum reference value were categorized as abnormally high.

Serum levels of C-reactive protein (CRP) were measured with an ELISA (LZ test Eiken CRP-HG; Eiken Kagaku, Tokyo, Japan), according to the manufacturer's instructions. Values >0.3 mg/dl were categorized as abnormally high.

The erythrocyte sedimentation ratio (ESR) was measured by Falco Biosystems (Kyoto, Japan). Values >10 mm/h for men and >15 mm/h for women were categorized as abnormally high.

The PEG-precipitable protein ratio (%), which represents the amount of γ -globulin plus proteins that bind to PEG nonspecifically, was calculated as follows: (total protein – protein in the supernatant after treatment with 12.5% PEG)/total PRL $\times$ 100. The protein concentrations were measured with a Micro BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions.

Serum levels of antibodies against cyclic citrullinated peptides (anti-CCP) were measured with Immunoscan CCPlus (Euro Diagnostica, Malmo, Sweden), according to the manufacturer's instructions. Samples with more than 25 U/ml of anti-CCP antibodies were defined as positive.

Digestion of PRL with MMP-3

MMP-3 was activated according to the manufacturer's instructions. Recombinant human MMP-3 (R&D Systems, Inc., Minneapolis, MN, USA) was activated at

20 μ g/ml in an assay buffer (50 mM Tris, 10 mM CaCl₂, 150 mM NaCl and 0.05% Brij-35, pH 7.5) containing 5 μ g/ml chymotrypsin. The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by adding 2 mM phenylmethylsulphonyl fluoride (PMSF). PRL (50 ng) was incubated with various amounts of the activated MMP-3 in 10 μ l assay buffer at 37 °C for 1 h and subjected to SDS-PAGE and western blotting.

SDS-PAGE and western blotting

MMP-3-digested PRL was heated at 100 °C in 125 mM Tris-HCl buffer (pH 8.3) containing 4 M urea, 2% SDS and 5% 2-mercaptoethanol for 3 min and electrophoresed on a 10% polyacrylamide gel containing 0.1% SDS. The proteins in the gel were electrotransferred onto a PVDF membrane (Bio-Rad Laboratories, Inc.) and incubated with anti-human PRL antiserum. After incubation with goat anti-rabbit immunoglobulin-peroxidase conjugate (Cosmo Bio, Co., Tokyo, Japan), the bands were visualized with ECL western blotting detection reagents (GE Healthcare, Buckinghamshire, UK) and detected by a lumino-image analyzer (LAS-4000 mini, Fuji Photo Film Co., Ltd., Kanagawa, Japan).

Statistical analysis

Values are expressed as means $\pm$ s.d. Statistical significance was evaluated with Student's unpaired *t*-test and the χ^2 test in StatMate III statistic software (ATMS, Tokyo, Japan). Correlation coefficients between variables were calculated by a linear regression analysis. Serum PRL concentrations and anti-CCP antibodies

Figure 1 Prevalence of macroprolactinaemia in patients with RA and control subjects. The prevalence of macroprolactinaemia is shown for young control subjects, elderly control subjects and patients with RA. The numbers shown in the bars indicate the number of subjects with macroprolactinaemia/total number of subjects in that group. The χ^2 test was used to compare the groups.

Table 1 Clinical characteristics and parameters of the study subjects.

	Young control	Elderly control	RA
Number of subjects	219	83	238
Women/men	185/34	69/14	200/38
Age	29.4 ± 2.6	59.4 ± 2.4	60.7 ± 12.5
Total PRL (mIU/l)	233.4 (63.0–882.0)	113.1 (45.5–273.0)	225.4* (77.0–654.5)
Free PRL (mIU/l)	149.1 (42.0–514.5)	73.2 (35.0–147.0)	141.8† (56.0–353.5)
MMP-3 (ng/ml)	49.8 ± 18.9	56.6 ± 21.8‡	120.6 ± 88.2§
ESR (mm/h)	ND	ND	27.0 ± 18.7
CRP (mg/dl)	ND	ND	0.76 ± 0.87
Anti-CCP antibody (U/ml)	ND	ND	293.4 (14.5–6023.3)

*Mean (95% CI). $P < 0.001$ compared with that of the age- and gender-matched elderly control subjects. † $P < 0.001$ compared with that of the age- and gender-matched elderly control subjects. ‡ $P = 0.045$ compared with that of the young control subjects. § $P < 0.001$ compared with that of the young and elderly control subjects.

were logarithmically transformed to get the distribution close to normal. The 95% CIs for ln-transformed data were determined and back-transformed. $P < 0.05$ was considered significant.

Results

Prevalence of macroprolactinaemia in RA

Fifteen patients with RA had macroprolactinaemia (15/238 patients; 6.3%). The prevalence among patients with RA was significantly ($P = 0.036$) higher than that in the gender-matched young control group (5/219 subjects; 2.3%), but not different ($P = 0.93$) from that in the age- and gender-matched elderly control group (5/83 subjects; 6.0%) (Fig. 1).

Serum total PRL concentrations were significantly higher in patients with RA (mean: 225.4 mIU/l, 95% CI: 77.0–654.5 mIU/l) than in the age- and gender-matched control subjects (113.1 mIU/l, 45.5–273.0 mIU/l; $P < 0.001$; Table 1). Serum free PRL concentrations were also significantly higher in patients with RA (141.8 mIU/l, 56.0–353.5 mIU/l) than in the age- and gender-matched control subjects (73.2 mIU/l, 35.0–147.0 mIU/l; $P < 0.001$; Table 1). Hyperprolactinaemia (serum total PRL concentrations > 525 mIU/l) occurred more frequently in RA patients (26/238 patients; 10.9%) than in the elderly control subjects (2/83 patients; 2.4%; $P = 0.018$).

We examined the levels of three indicators of RA disease activity: MMP-3, CRP and ESR. Increases in the levels of these markers reflect greater severity of inflammation and joint destruction. We found that serum MMP-3 levels were elevated in 124 patients (52.1%) with RA. Patients with elevated MMP-3 levels had a significantly higher prevalence of macroprolactinaemia (12/124 patients; 9.68%; $P = 0.025$) than those with normal MMP-3 levels (3/114 patients; 2.63%; Fig. 2). As shown in Table 1, serum MMP-3 levels in the elderly control subjects (56.6 ± 21.8 ng/ml) were significantly ($P = 0.045$) higher than those in the young control subjects (49.8 ± 18.9 ng/ml). Serum CRP and ESR levels were elevated in 107 and 142

patients respectively. However, the prevalence of macroprolactinaemia was not significantly different between patients with and without elevations in the levels of these inflammatory markers (Fig. 2).

Digestion of PRL with MMP-3

PRL (50 ng) was digested with various amounts of MMP-3 (1, 2, 4, 8 and 20 ng) in 10 µl assay buffer. As shown in Fig. 3, digestion of the 23 kDa PRL molecule with MMP-3 produced 14, 15 and 16 kDa vasoinhibins. As the amount of MMP-3 increased, the proportion of larger vasoinhibin species decreased and that of smaller species increased. The 23 kDa PRL band migrated a little slowly when treated with MMP-3 with the cause being unknown.

Figure 2 Prevalence of macroprolactinaemia in patients with RA who exhibited high or low levels of inflammatory markers. The prevalence of macroprolactinaemia was examined in patients with RA who exhibited high (+) or low (–) disease activity, based on serum levels of MMP-3, CRP and ESR. The numbers shown in the bars indicate the number of patients with macroprolactinaemia/total number of patients in that group. The χ^2 test was used to compare the groups.

Figure 3 Digestion of PRL with MMP-3. PRL (50 ng) was digested with various amounts of MMP-3 (1, 2, 4, 8 and 20 ng) in 10 μ l assay buffer and analysed by SDS–PAGE and western blotting. The lane (–) indicates PRL incubated in the absence of MMP-3. The positions of the molecular weight markers are indicated by arrows.

Relationship between macroprolactin and γ -globulin and anti-CCP antibody

The levels of PEG-precipitable protein, which includes γ -globulin and proteins that bind to PEG non-specifically, ranged from 14.3 to 55.0% ($32.5 \pm 7.5\%$) in patients with RA. The levels of PEG-precipitable protein were not significantly correlated with those of the PEG-precipitable PRL ($r=0.02$; Fig. 4a). The levels of PEG-precipitable protein were not significantly different between RA patients with and without macroprolactinaemia (30.3 ± 8.6 and $32.6 \pm 7.5\%$ respectively, $P=0.26$). Gel filtration chromatography confirmed that all 15 patients with RA and macroprolactinaemia had significantly elevated ratios of PRL molecules larger than 150 kDa (macroprolactin; $59.7 \pm 21.5\%$, range 26.9–94.2%) than those with RA but without macroprolactinaemia ($n=10$; $2.9 \pm 2.3\%$, range 0–7.1%, $P<0.001$; Fig. 4b). Anti-CCP antibody was positive (>25 U/ml) in 212 of 238 (89.1%) patients with RA. However, there was no

significant correlation between the levels of anti-CCP antibody and the levels of PEG-precipitable PRL ($r=0.09$; Fig. 4c).

Discussion

Macroprolactin is a heterogeneous molecule, but it mainly comprises a complex of PRL with IgG, particularly anti-PRL autoantibodies. The results of this study suggest that elevated levels of MMP-3 might contribute to the generation of anti-PRL autoantibodies in some patients with RA.

MMP-3 is an enzyme secreted by synovial fibroblasts and chondrocytes that degrades connective tissue matrix components. Increased levels of MMP-3 in serum and synovial fluids are frequently observed in RA. MMP-3 levels reportedly reflect the intensity of intrasynovial inflammation and joint destruction (11, 12). MMP-3 has a broad spectrum of substrate specificities, and PRL is one of its substrates. MMP-3 cleaves the PRL molecule at Leucine¹⁵⁶–Glutamine¹⁵⁷ and at Alanine¹¹¹–Isoleucine¹¹². This produces N-terminal fragments of 17 and 12 kDa respectively (13). These N-terminal fragments of the PRL molecule are termed 'vasoinhibins' because they inhibit angiogenesis by suppressing growth factor-induced endothelial cell proliferation (14). In contrast, the parental 23 kDa PRL molecule lacks such an effect on endothelial cells. In this study, we confirmed that MMP-3 could digest the PRL molecule and produce vasoinhibins with various molecular species. We tried to identify the presence of vasoinhibins in the sera collected from

Figure 4 Relationship between the levels of macroprolactin and those of γ -globulin and anti-CCP antibody. (a) Correlation between the ratios of the PEG-precipitable protein (γ -globulin) and PEG-precipitable PRL (macroprolactin) in patients with RA. Macroprolactinaemia is indicated by the shaded area (PEG-precipitable PRL ratio $>60\%$). (b) Representative gel filtration chromatographic profiles of serum PRL of RA patients, young control subjects, and elderly control subjects with and without macroprolactinaemia. Macroprolactin is represented by the 150 kDa peak and monomeric PRL is represented by the 23 kDa peak. (c) Correlation between the levels of anti-CCP antibody and PEG-precipitable PRL (macroprolactin) in patients with RA. Macroprolactinaemia is indicated by the shaded area (PEG-precipitable PRL ratio $>60\%$).

RA patients with elevated MMP-3 levels. However, we were unable to obtain definitive results because the volume of the clinical samples was not sufficient and the serum PRL levels were not high enough to be detected by western blotting.

The present finding that the prevalence of macroprolactinaemia in RA patients with elevated serum MMP-3 levels was higher than that in those with normal levels might indicate that MMP-3 is involved in the pathogenesis of macroprolactinaemia in some RA patients. The cleavage of the PRL molecule may expose new epitopes that elicit an immune response and production of anti-PRL autoantibodies. These autoantibodies may cross-react with the parental PRL molecule and generate macroprolactin. However, it is unlikely that MMP-3 is an independent cause of macroprolactinaemia, in general, because the prevalence of macroprolactinaemia in RA patients was higher than that in the young control subjects but similar to that in the age- and gender-matched control subjects. If MMP-3 is an independent cause of macroprolactinaemia, the effect of elevated MMP-3 levels should be added to that of ageing, resulting in a higher prevalence of macroprolactinaemia in RA patients than in the age-matched control subjects. Serum MMP-3 levels in the elderly control subjects were found to be higher than those in the young control subjects, in accordance with the previous report that serum MMP-3 values were positively correlated with age (16). Some unknown factor operating upstream of MMP-3 and ageing may be involved in the pathogenesis of macroprolactinaemia.

Macroprolactinaemia screening is performed with 12.5% PEG, which precipitates γ -globulin. One study has shown that some patients with high serum γ -globulin levels had pseudomacroprolactinaemia; thus, no macroprolactin was observed on gel chromatography, despite an increase in the PEG-precipitable PRL ratio (17). Because RA is a chronic inflammatory disorder characterized by elevated γ -globulin levels, some patients with RA might be expected to exhibit pseudomacroprolactinaemia. However, in the present study, we did not observe any difference in the PEG-precipitable protein (γ -globulin plus proteins that bind to PEG nonspecifically) levels between patients with and without macroprolactinaemia. Furthermore, there was no correlation between the PEG-precipitable protein and PEG-precipitable PRL levels in our patients. Moreover, we confirmed that all 15 patients with RA and macroprolactinaemia (judged by the PEG method) actually had macroprolactinaemia, based on gel chromatography. This suggests that such levels of γ -globulin present in RA may not directly cause pseudomacroprolactinaemia.

Several studies have shown that serum PRL levels in patients with RA are elevated (18, 19). The present study demonstrated that serum total and free PRL levels in RA patients were significantly higher than those in the age- and gender-matched control subjects. Since PRL reportedly induces the maturation of T cells, develops

antigen-presenting cells, enhances immunoglobulin production and breaks down B-cell tolerance to self via its anti-apoptotic action (20), the increase in serum PRL levels may contribute to the development of RA.

In the early literature on macroprolactinaemia, some associations have been reported between macroprolactinaemia and autoimmune disorders (21, 22). However, later studies that included larger patient cohorts have revealed that, although many patients with macroprolactinaemia possessed antithyroglobulin and thyroid peroxidase antibodies, the prevalence of these autoantibodies was not significantly different from that observed in the controls (3, 4, 9). Wallace *et al.* (23) followed 51 patients with macroprolactinaemia for 10 years, and they did not observe any symptoms suggestive of the development of autoimmune conditions. Anti-CCP antibody is a specific autoantibody in RA, and the titre of anti-CCP antibody is related to RA severity (24). In the present study, we found no correlation between the ratios of macroprolactin and the levels of anti-CCP antibodies. The results of the present study indicate that the development of anti-PRL autoantibodies might not be associated with that of RA-specific antibodies.

In summary, our findings indicate a possibility that MMP-3 might be involved in the development of macroprolactinaemia in some patients with RA and that such post-translational modifications of the PRL molecule may lead to the increased prevalence of macroprolactinaemia in elderly subjects.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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