New ELISA System for Myeloid-related Protein Complex (MRP8/14) and Its Clinical Significance as a Sensitive Marker for Inflammatory Responses Associated with Transplant Rejection

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Background: C-reactive protein (CRP), a useful marker for inflammatory diseases, is not always sensitive to inflammatory reaction in the liver or other tissues. The aim of this study was to develop a sensitive and specific method for detecting inflammatory responses associated with transplant rejection.

Methods: We developed a new, highly sensitive ELISA system for the measurement of serum human myeloid-related protein complex (MRP8/14), using monoclonal antibodies against MRP8/14, and applied it to specimens obtained from patients undergoing small intestine or liver transplantation.

Results: This assay could detect MRP8/14 concentrations as low as 2 μg/L. Within-run CVs were 3.7–6.1% and between-day CVs were 5.6–8.7% for MRP8/14 concentrations of 117–3300 μg/L. Mean recovery was 104% (range, 80–128%). We observed a marked increase in serum MRP8/14 postoperatively in most recipients of transplants, followed by an increase in CRP 1–7 days after the increase in the complex. The increase in serum MRP8/14 occurred simultaneously with permeation of lymphocytes into the transplanted tissues as a result of rejection of the graft tissues.

Conclusions: Accurate measurement of serum MRP8/14 provides a useful clinical diagnostic method tool for detecting inflammation associated with rejection of transplanted tissues.

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Human granulocytes and monocytes are two major effector cells involved in inflammatory processes in the body. To acquire the potential ability of inactive granulocytes and monocytes, cells must generally be activated intracellularly in a calcium-dependent manner or by other factors (1–3). Excessive activation of these cells may, however, induce pathophysiologically unfavorable conditions in many acute and/or chronic inflammatory diseases (4,5). The myeloid-related protein complex (MRP8/14),4 consisting of two subunits with low molecular mass (8 and 14 kDa), is a member of the S100 family and contains two calcium-binding sites per molecule (2,6–8). The two subunits are expressed in activated human granulocytes and monocytes in the inflammatory phase (1,3,8–10). Both proteins form covalently associated complexes in a calcium-dependent manner (11,12). Translocation of the complexes from the cytosol to membrane is induced under increased intracellular calcium concentrations and correlates with the inflammatory action of granulocytes and monocytes (3,13). Thus, MRP8 or MRP14 and their heterodimeric complex, MRP8/14, seem to be key proteins in cell-cycle regulation and cell activation with calcium to motivate phagocytosis (14–16). The calcium-induced change in the complex pattern of these two proteins is important for their biological function.
Odink et al. (1), on the other hand, reported that neither MRP8 or MRP14 was detectable in resident macrophages in sections of healthy human tissue, but in acutely inflamed tissue, such as in gingivitis, psoriasis, neurodermatitis, and erythrodermia, MRP14 was present in macrophages of the perivascular infiltrate in 15 of 32 cases analyzed, whereas MRP8 was always absent. Both MRP8 and MRP14 are involved in acute or chronic inflammation. Recently, we purified MRP8/14 protein from human leukocytes, prepared specific monoclonal antibodies against the complex, and used these antibodies to develop an ELISA system.

To date, there have been few reports on the clinical importance of MRP8/14 as a marker of inflammatory reaction associated with transplant rejection in graft tissues, particularly in the acute phase (17, 18). The present study was based on our hypothesis that MRP8/14 could serve as a useful marker for acute inflammation associated with rejection in the grafted tissues.

Materials and Methods

Materials

We obtained 96-well polycarbonate plates for ELISA and Block-Ace™ solution for blocking the plate from Dainippon Pharmacology Co. Ltd. CM Sephadex C-50 and molecular weight markers were from Pharmacia. Poros HS was from the Applied Biochemistry Co. Ltd. Purified recombinant human liver-type arginase (ARG) was used as a calibrator for the ELISA. All analytical reagents were obtained from Nacalai Tesque Co. Ltd.

Preparation of Human Leukocyte Lysate

Human whole blood was anticoagulated with ACD-A solution (22 g/L sodium citrate, 8 g/L citrate, and 22 g/L glucose in distilled water). The blood and plasma gel solution (30 g/L gelatin and 9 g/L NaCl) was gently mixed at a ratio of 6:1 and then incubated at 37 °C for 1 h. The supernatant was collected in a 50-mL plastic tube with a plastic pipette and then centrifuged at 1500g for 5 min at room temperature. The supernatant was discarded. We added 15 mL of 10 mmol/L Tris-HCl (pH 7.4) containing 1 g/L NH₄Cl to the remaining cell fraction to hemolyze contaminated erythrocytes, mixed the cell fraction gently with the same plastic pipette, and then incubated it at 37 °C for ~5 to 7 min. After termination of hemolysis, the cells were washed three times with Hank’s balanced salts solution (HBSS). The recovered leukocytes were resuspended in ~30 mL of 10 mmol/L Tris-HCl (pH 7.4) buffer solution. After sonication, the solution was centrifuged at 11 000g at 4 °C for 30 min; the supernatant was collected and then applied to a CM Sephadex C-50 column.

Purification of MRP8/14 Protein Complex

Partial purification of MRP8/14 was achieved with use of CM Sephadex C-50 (19 × 100 mm) and Poros HS [100 × 4.6 mm (i.d.); 20 μm; Vision Workstation FPC] columns. The buffer solution used was 10 mmol/L Tris-HCl (pH 7.4). Proteins were eluted with a linear gradient of 0–1.0 mol/L NaCl in the same buffer. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to confirm that the fractions contained proteins with molecular masses of ~26 kDa, as described by Edgeworth et al. (8). Finally, affinity-purified MRP8/14 proteins were obtained with use of an anti-MRP8/14 complex monoclonal antibody (Mo2B9)-Sepharose 4B column according to the procedure described previously (20). In addition, MRP8 and MRP14 proteins were purified electrophoretically. Briefly, SDS-PAGE (15% gel) of partially purified MRPs (~90%) was carried out in the absence of 2-mercaptoethanol (2-ME). After SDS-PAGE, the proteins in the gel were stained with Coomassie Brilliant Blue. Two 3-mm gel slices corresponding to 8 and 14 kDa were carefully cut out and separately placed in seamless cellulose tubing for dialysis, together with an adequate volume of the buffer solution for SDS-PAGE. Electrophoresis was again carried out in sufficient volume of the same buffer, using an apparatus for electrophoresis of DNA. MRP8 and MRP14 were electrophoretically released from the gel slices into the buffer solution. MRP8 or MRP14 in the buffer was concentrated and kept refrigerated until use.

Production of Monoclonal Antibodies for Anti-MRP8/14 Protein and Their Specificities

Anti-MRP8/14 complex monoclonal antibodies were prepared as described previously (21). These antibodies were biotinylated with NHS-biotin (Pierce) according to commercial instructions for application to ELISA and stored refrigerated as the second antibody until use. The specificities and affinities of these antibodies were examined by Western blot analysis that was carried out at room temperature using nitrocellulose membranes, anti-MRP8/14 complex monoclonal IgGs, and commercially available anti-mouse IgG IgG-horseradish peroxidase (HRP) conjugate as described previously by Towbin et al. (22). Antibody-bound protein was visualized with use of a color reagent [3–5 mg of 3,3′-diaminobenzidine tetra- chloride in 20 mL of 0.1 mol/L Tris-HCl (pH 7.6) and 0.1 mL/L hydrogen peroxide]. Briefly, the crude MRP8/14 protein (~200 μg of protein) was added to each lane (see Fig. 1, A, C, and D). The first antibodies (Mo1B12, Mo2B9, Mo3D2, and Mo3F3), adequately diluted, were used to examine their specificities for the MRP8/14 complex. The MRP8, MRP14, and MRP8/14 proteins, which were electrophoretically purified from 15% gels by SDS-PAGE in the absence of 2-ME, were used (see Fig. 1, B–D). These MRPs were used for SDS-PAGE in the presence and the absence of 2-ME; the proteins in the gels were then transferred on nitrocellulose membranes electrophoretically.
The purified MRP8/14 complexes were subjected to SDS-PAGE (15% gel) under reducing conditions with 2-ME. Two subunits of MRP8 and MRP14 in the polyacrylamide gel were transferred to a PVDF membrane (Sequiblot™; Bio-Rad Laboratories) and used for amino acid sequence analysis. Amino acid sequence analysis of the purified MRP8/14 was carried out to investigate the homology with MRP8/14 reported by Odink et al. (1). The peptide sequences of both MRP8 and MRP14 subunits purified in this study were identical to those of MRP8 and MRP14, respectively, reported by Odink et al. (1) (data not shown).

ELISA PROCEDURES FOR MRP8/14 COMPLEX AND ARG
The basic examination of optimal conditions for the ELISA was carried out in detail as described previously (21). Concentrations of 0.166 and 0.834 mg/L for the first and second antibodies, respectively, were the most effective for the ELISA system (data not shown). A 96-well polycarbonate plate coated with the first antibody (Mo2B9) was used for ELISA. We added 100 μL of diluent solution to each well; we then added 25 μL of the serum sample, previously diluted 41-fold with Block-Ace diluted 10-fold with distilled water (a working Block-Ace solution), or the MRP8/14 complex calibrator solution and mixed the plate for 15 s with a plate mixer. The plate was incubated for 1 h to allow the immunologic reaction to proceed (first incubation). After the plate was washed five times with washing buffer solution, we added 100 μL of the F(ab')2-biotin conjugate (second antibody; Mo3D2 in the working Block-Ace solution, containing 0.5 g/L thimerosal) and incubated the plate for 1 h (second incubation). After washing the plate as above with the same washing solution, we added 100 μL of streptavidin-HRP conjugate diluted 1500-fold with the working Block-Ace solution and incubated the plate for 30 min (third incubation). Finally, we determined HRP activity colorimetrically by the method of Ikemoto et al. (20) as described previously. If the patient’s serum contains a high concentration of MRP8/14, the serum samples should be diluted adequately with a solution containing 10 g/L bovine serum albumin. A lower dilution of the sample can be used, however, to shorten the time for the first and second incubations in the ELISA system. ELISA for ARG was carried out as described previously (21). Using the ELISA system, we determined the ARG concentrations in the sera of a large number of liver recipients and a few recipients receiving small intestine transplants.

Results
We performed SDS-PAGE (15% gel) on partially purified MRP8/14 protein complex from human leukocytes, without a reducing agent, and then performed Western blot analysis using 16 monoclonal antibodies. The representative results are presented in Fig. 1. Of these antibodies, regardless of the crude MRP8/14 sample, two antibodies (Mo2B9 and Mo3D2) revealed a single band, indicating the specificity of these antibodies for the MRP8/14 complex (Fig. 1A, lanes 2 and 3). The reactivity of Mo2B9 with MRP8/14 was a little stronger than that of Mo3D2. SDS-PAGE of MRP8, -14, and -8/14 was carried out in the absence (Fig. 1B, lanes M, 1, 2, and 3) and the presence (Fig. 1B, lanes 4, 5, and N) of 2-ME. MRP8/14 (Fig. 1B, lane 1) was reduced to two smaller subunits corresponding to MRP8 and MRP14 (Fig. 1B, lane 4), respectively, indicating that the 26-kDa protein is a MRP8/14 complex comprising the MRP8 and -14 subunits. For comparison, the MRP8 band under reducing conditions was also present (Fig. 1B, lane 5). However, the gel in Fig. 1A does not necessarily show that Mo2B9 and Mo3D2 recognize different epitopes of the MRP8/14 molecule. To elucidate the specificities of the two antibodies for their different epitopes in the MRP8/14 complex, we performed an inhibition assay (Fig. 1C).

The reactivity of Mo2B9-HRP as second antibody was completely inhibited when we used Mo2B9 as the first antibody (Fig. 1C, lane 1), but when Mo3D2-HRP was used as the second antibody with Mo2B9 as the first antibody, we observed a strong positive band (Fig. 1C, lane 2). Similar to Mo2B9, the reactivity of the Mo3D2-HRP was completely inhibited when we used Mo3D2 as the first antibody (Fig. 1C, lane 3), but when we used Mo2B9-HRP as the second antibody and Mo3D2 as the first antibody, we observed a positive band (Fig. 1C, lane 4). These facts indicate that the epitope detected by Mo3D2 was different from that detected by Mo2B9. In addition, both of the antibodies reacted with MRP8/14 protein specifically but not at all with the individual subunits, MRP8 or MRP14 (Fig. 1D). It therefore is likely that these antibodies recognized the higher dimensional structure of MRP8/14 complex characterized by special epitopes and recognized different epitopes in the MRP8/14 molecule. On the basis of these results, we used Mo2B9 and Mo3D2 as the first and the second antibodies, respectively, to develop an ELISA system for MRP8/14.

To examine the specificity of the 16 monoclonal antibodies for the MRP8/14 complex, we performed Western blotting. When we used a crude extract of the MRP8/14 complex from human leukocytes, the antibody Mo2B9 reacted most specifically with the MRP8/14 complex, whereas the antibody Mo3D2 had the highest affinity for the complex, revealing a single, strongly positive band by Western blotting (data not shown). In addition, we examined which pair of monoclonal antibodies was the best combination for a sandwich ELISA and found that the sensitivity and specificity were highest when we used Mo2B9 as the first antibody and previously biotinylated Mo3D2 as the second antibody, with a fixed concentration of streptavidin-HRP conjugate adequately diluted with 10 g/L bovine serum albumin in the working Block-Ace solution (Fig. 2). The ELISA enabled us to measure MRP8/14 protein at concentrations as low as 2 μg/L in serum (Fig. 2) and was linear to 873 μg/L.
We assessed the reproducibility of the method using purified MRP8/14 calibrator and pooled sera from healthy individuals and patients undergoing liver or small intestine transplantation. When we used the MRP8/14 calibrator (936 μg/L) and the pooled serum (312 μg/L), the within-day CVs were 3.7% and 6.1%, respectively (n = 44). When we used the pooled sera of 3300 and 312 μg/L, the between-day CVs were 5.6% and 8.7%, respectively (n = 35). The mean recovery of endogenous plus exogenous MRP8/14 was 104% (range, 80–128%). The MRP8/14 concentrations in the sera obtained from 85 healthy individuals (32 males and 53 females; mean age, 19 years; range, 18–25 years), were 52–468 μg/L [mean (SD), 260 (104) μg/L].

To assess the clinical usefulness of our new ELISA system for MRP8/14, we examined sera from 225 patients undergoing either small intestine or liver transplantation from living related donors. Postoperative changes in serum MRP8/14 concentrations in one representative case are presented. For comparison, the serum concentrations of ARG, aspartate aminotransferase (AST), alanine aminotransferase (ALT), C-reactive protein (CRP), and other markers were also measured.

A female patient (7 years of age) with congenital intestine absorption disorders underwent a partial small intestine transplantation from her mother. Approximately 6 months after the operation, a dramatic increase in the patient’s serum MRP8/14 was observed, followed by an
increase in CRP (Fig. 3). Approximately 1 month after the initial increase, transplantation of a part of the small intestine was repeated (indicated by a vertical arrow in Fig. 3). However, rejection occurred progressively, and the patient died ~1 year later. At 6 months after the repeat transplantation, serum MRP8/14 increased markedly to 50 times the upper limit of the reference interval, with two prominent peaks. The first, and less prominent, peak emerged ~4 months before death. The concentration continued to increase with repeated fluctuations. At the terminal stage, the second prominent peak increased to 100 times the upper limit of the reference interval just before the patient’s death. At the time points of the three peaks, rejections in the grafted tissues were confirmed histochemically (data not shown). Thus, the time periods in which rejection appeared coincided with the prominent MRP8/14 peaks. CRP, on the other hand, increased only before the patient’s death, to 40 times the upper limit of the reference interval.

**Discussion**

In the present study, we described a new ELISA system for measuring the MRP8/14 protein and presented data supporting its clinical usefulness. The value of serum MRP8/14 as an indicator of acute inflammation in transplanted tissues is based on its origin and characteristics as a protein in relation to acute inflammation. Two new aspects of MRP8/14 as a useful marker for acute inflammation associated with transplant rejection were found in the present study: one is that the appearance of MRP8/14 precedes that of CRP (Fig. 3); the other is that fluctuations in serum MRP8/14 concentrations are much more sensi-
tive for acute inflammatory responses associated with rejection in the transplanted tissues than are those of the other markers, such as CRP, AST, and ALT (Fig. 3).

CRP has been recognized as a good marker for inflammation by many clinicians and reported to be produced in the liver by stimulation of several cytokines, such as interleukin-1, interleukin-6, and tumor necrosis factor-α, in a complex manner (23). It is also generally accepted that CRP becomes detectable in the blood at least 2 to 3 days after the stimulation. On the other hand, MRP8/14 complex is likely to be simultaneously produced in human granulocytes in the acute inflammatory phase because MRPs (MRP8 and MRP14), particularly MRP14, are expressed exclusively in human activated granulocytes, mainly neutrophils, in response to acute inflammatory reaction at the early stage (1, 13). This property of the MRP8/14 complex is a potential benefit that is more advantageous for detecting acute inflammation than is CRP. Although the mechanism for leakage of the MRP8/14 complex from human activated granulocytes is unclear, it is different from that of CRP (3). Differences in the kinetics of MRP8/14 and CRP in serum, i.e., differences in the fluctuations of their concentrations in serum, provide another useful marker for inflammation caused by rejection.

There were no significant increases in AST, ALT, and ARG throughout our monitoring of the representative case of small intestine transplantation, indicating no damage to the liver and other organs, such as the heart and kidney (24, 25). At 1, 3, and 6 months before the patient’s death, however, high concentrations of MRP8/14 were detected in her serum, suggesting that a severe inflammatory reaction occurred elsewhere in nonhepatic tissues (Fig. 3). Indeed, transplant rejection was confirmed microscopically in the transplanted tissues at each time point, as indicated by the asterisks in Fig. 3 (data not shown). These facts support our hypothesis that the overexpression of MRP8/14 in human granulocytes in the inflammatory phase and its appearance in the serum is probably the result of inflammatory reactions associated with rejection in the transplanted tissues. Thus, the increase in CRP was not necessarily concomitant with that in MRP8/14 and may be attributable to bacterial infection in the recipient (18). Therefore, serum MRP8/14 seemed to be more sensitive for inflammatory responses associated with transplant rejection than was CRP. Throughout the whole course, serum MRP8/14 tended to increase earlier and rather independently in comparison with CRP, which indicated that MRP8/14 is not a critical marker as an ultimate index for death in recipients, but rather serves as a prospective marker to detect acute immune changes relating to rejection or inflammation occurring in the body.

Some roles of MRP8 and MRP14 in the cells have been reported (6, 11, 14, 26), whereas those of the MRP8/14 complex are poorly understood. Although the reason that MRP8 is covalently associated with MRP14 at the time of acute inflammation remains unclear, such an association may enhance their potential pathophysiologic functions that down-regulate acute inflammatory reaction in the transplanted tissues in a complex manner.

In conclusion, we described the possibility of the MRP8/14 complex being a reliable marker for inflammatory reactions associated with rejection in transplanted tissues. In future studies, the expression mechanism of MRP8/14 and its functions in human activated granulocytes, and the relationship between MRP8/14 and CRP should be investigated.

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