Aggregated rmMPO was prepared by mixing and incubation with PBS for 1 h at 4°C. The aggregates were intensively washed with PBS until protein was undetectable in supernatant by UV absorption and incubated with 10 mg/ml anti-rmMPO IgG for 1 h at 4°C with agitation. As a control, the same amount of anti-rmMPO IgG was incubated in the absence of aggregated rmMPO. The mixture was centrifuged and supernatant was used for experiments. $F(ab')_2$ fragments were prepared using an ImmunoPure $F(ab')_2$ preparation kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Detection of ICAM-1 in mGEC (Cell ELISA)

The mGEC were plated on collagen-coated 96-well plates (Iwaki) at a density of 3×10^3 cells/well. At confluency, the cells were washed once with warmed HBSS and incubated for 1 h in RPMI-1640 medium containing 1% heat-inactivated FBS (assay medium). Anti-rmMPO IgG or control rabbit IgG diluted in assay medium was added to the wells and incubated for indicated periods, then the cells were washed with warmed PBS three times and fixed in 0.2% glutaraldehyde for 5 min at 4°C. For the blocking experiment, a neutralizing rat monoclonal antibody to TNF-α (BD Pharmingen, San Diego, CA) was included in assay medium throughout the incubation period. Non-specific binding was blocked by incubation with 1% BSA in PBS overnight at 4°C, followed by incubation for 1.5 h at room temperature with 0.5 μg/ml rat anti-mouse ICAM-1 monoclonal antibody (KAT-1; Chemicon, Temecula, CA). The primary antibody was detected by incubation for 1.5 h at room temperature with an alkaline phosphatase-conjugated anti-rat IgG antibody (1:2000, Bio-Rad). The bound secondary IgG was measured by the same procedure as aforementioned.

Detection of anti-endothelial cell antibodies

Anti-endothelial activity of antibodies used in this study was measured by the methods previously described by Carvalho *et al.* [20] with modification. mGEC were seeded onto a collagen-coated 96-well plates (Iwaki) at a density of 3×10^3 cells/well. The cells were fixed with 0.2% glutaraldehyde for 5 min at 4°C. The plates were blocked overnight with 1% BSA in PBS at 4°C and then incubated for 2 h with various concentrations of anti-rmMPO IgG or control IgG. The bound IgG was detected by an alkaline phosphatase-conjugated anti-rabbit IgG antibody (1:4000, Bio-Rad) with subsequent quantification of phosphatase using *p*-nitrophenyl phosphate as aforementioned. To measure the reactivity of anti-rmMPO IgG to live mGEC, the antibodies were first incubated with the live mGEC in the assay medium and then fixed with 0.2% glutaraldehyde. The bound IgG was measured by the same procedure as described before.

Immunoprecipitation

The anti-rmMPO or control rabbit IgG were bound to protein A Sepharose beads in lysis buffer [20 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), pH 7.5] containing 0.1% BSA for 2 h at 4°C and washed several times with lysis buffer. Cells were lysed with lysis buffer and incubated with the antibody-bound protein A sepharose beads for 2 h at 4°C. The sepharose beads were washed three times with lysis buffer and bound proteins were solubilized by boiling in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The protein samples were electrophoresed on 5–20% gradient gel and visualized by SYPRO Ruby staining (Molecular Probes, Eugene, OR).

Reverse transcript polymerase chain reaction (RT–PCR)

Total RNA was extracted using an ISOGEN (Nippongene, Tokyo, Japan), according to the manufacturer's instructions and quantified by UV absorption. One microgram of total RNA was reverse transcribed using ReverTra Ace-alpha (TOYOBO, Osaka, Japan). A semi-quantitative RT–PCR was used for the estimation of TNF-α mRNA expression. One microlitre of the resulting cDNA was used for PCR in 20 μl of Ex *Taq* buffer containing 0.2 mM dNTP mix, 0.5 μM of each primer and 25 U/ml Ex *Taq* polymerase (all reagents were purchased from TaKaRa Bio Inc.). Primer sequences for specific cDNA fragments were TNF-α: forward 5′-ctactgaacttcggggtgatcg-3′, reverse 5′-aagtctaagtacttgggca gattgac-3′, β-actin: forward 5′-atctggcaccacaccttctacaat gagctgcg-3′, reverse 5′-catcgtactcctgcttgctgatccacatctgc-3′. The amplification reactions were allowed to proceed for 30 cycles for TNF-α or 20 cycles for β-actin consisting of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s. PCR products (10 μl) were electrophoresed on 2% agarose gels and stained with ethidium bromide. mRNA expression of ICAM-1, VCAM-1 and E-selectin was quantified by using a real-time RT–PCR. First-strand cDNA was synthesized as described above. One microlitre of cDNA samples was used for the PCR reaction and analysed by the ABI Prism 7000 Sequence Detection System (Applied Biosystems) using Taqman 2X Universal PCR Master Mix and Applied Biosystems Assays-on-Demand primers and Taqman probe sets specific for mouse ICAM-1, VCAM-1 and E-selectin, according to the manufacturer's instructions. A non-template control was included for each target analysed. Relative quantification of all targets was calculated by using the comparative cycle threshold method [26]. The levels of gene expression were standardized with those of the glyceraldehyde 3-phosphate dehydrogenase.

Immunofluorescence microscopy

Both mGEC and MC were plated on collagen-coated or non-coated glass coverslips (22 × 22 mm) in 35 mm culture dishes, respectively. The confluent monolayer was fixed in ice-cold methanol and blocked with 1% BSA in PBS overnight at 4°C. Rat monoclonal antibody to CD31 (MEC13.3, BD Pharmingen), or mouse monoclonal antibody to desmin (Sigma) diluted 1:100 in 1% BSA in PBS, were added onto each coverslip and the samples were incubated for 1 h at room temperature. The coverslips were washed three times with PBS and further incubated for 1 h at room temperature in FITC-conjugated goat anti-rat IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for CD31 or Alexa Fluor 488-conjugated anti-mouse IgG antibody (Molecular Probes) for desmin. The coverslips were washed and observed by

fluorescence microscopy. For the detection of ICAM-1, mGEC were incubated for 6 h with either 100 μg/ml anti-rmMPO or control IgG, fixed and immunostained for ICAM-1 using primary rat anti-mouse ICAM-1 monoclonal antibody and secondary FITC-conjugated goat anti-rat IgG antibody.

Statistical analysis

All data are expressed as mean ± SD. For individual comparisons, Student's *t*-test or Welch's corrected *t*-test, where appropriate, were used and differences with $P < 0.05$ were considered significant.

Results

Characterization of the primary mGEC

The population of the cells isolated from mice was characterized as endothelial cells by homogeneous monolayer of phase contrast image (Figure 1A) and positive staining with anti-CD31 (Figure 1B) in the junctional area and negative with anti-desmin (Figure 1C). The negative staining with anti-desmin indicates that there was little contamination of mesangial cells in the primary culture. In addition, RT–PCR analysis confirmed that mRNA of CD31 was expressed in the mGEC, but not in the mesangial cells (data not shown).

Up-regulation of adhesion molecule expression by anti-rmMPO IgG

To determine the direct effect of anti-rmMPO IgG on endothelial cell function, transcription levels of adhesion molecules in anti-rmMPO IgG-treated mGEC were examined. As shown in Figure 2A, quantitative RT–PCR revealed increases in mRNA levels of ICAM-1, VCAM-1 and E-selectin upon treatment with anti-rmMPO IgG in a dose-dependent fashion. The presence of 100 μg/ml of anti-rmMPO IgG in the culture medium increased ICAM-1, VCAM-1 and E-selectin transcripts in mGEC by 12.5, 7.5 and 10.5-fold, respectively. The control cells treated with normal rabbit IgG did not show any significant changes in the mRNAs of these adhesion molecules. Since ICAM-1, showed the highest increase in mRNA expression by anti-rmMPO IgG and, is a counter receptor for CD11b/CD18 integrins expressed abundantly in neutrophils, the following experiments were particularly focused on this molecule. The dose-dependent increase in ICAM-1 protein level in the anti-rmMPO IgG-treated mGEC was further confirmed by ELISA (Figure 2B). The mGEC treated

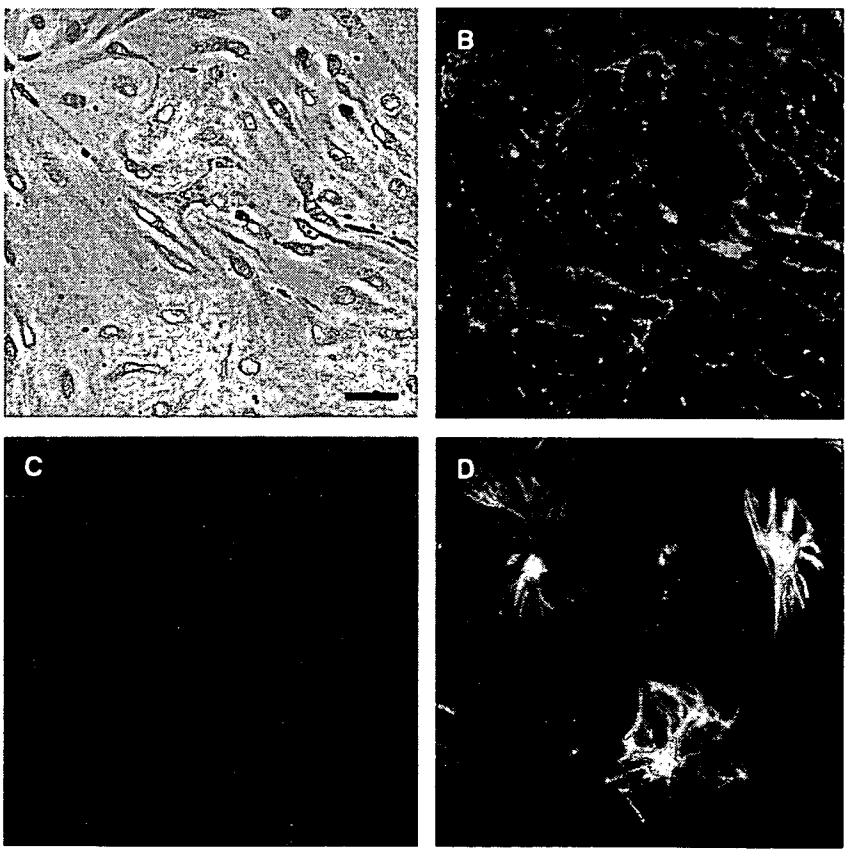

Fig. 1. Primary culture of mouse glomerular endothelial cells. Isolated mGEC were cultured on coverslips and phase contrast image (**A**) and immunofluorescence image for CD31 (**B**) were obtained in the same field. The mGEC were negatively stained by anti-desmin (**C**). Mesangial cells stained for desmin (**D**) served as a positive control. Bar = 20 μm.

with 100 μg/ml anti-rmMPO IgG for 6 h doubled the cellular ICAM-1 level and this condition was used for the following studies. In this condition, the increased expression of ICAM-1 by anti-rmMPO IgG was evident as shown by immunofluorescence microscopy for ICAM-1 (Figure 2C). In terms of cell morphology, there was no significant difference between anti-rmMPO IgG-treated cells and control normal IgG-treated cells (data not shown).

Involvement of rmMPO-specific antibodies in up-regulation of ICAM-1 expression in mGEC

To rule out the possibility of enhanced adhesion molecule expression by some contaminated substances, various concentrations of anti-rmMPO IgG were incubated with or without protein A sepharose beads for 1 h at 4°C and then the supernatants were used for assays. Anti-rmMPO activity was almost diminished at any concentration by incubation with protein A sepharose beads (Figure 3A). Similarly, the expression of ICAM-1 returned to control level by the depletion of IgG (Figure 3B), indicating that the enhancement of the ICAM-1 expression was induced only by the IgG molecules in anti-rmMPO IgG.

Since the polyclonal antibodies to rmMPO were used in this study, to rule out the up-regulation by non-specific IgGs, the experiment was conducted to confirm that the effects were induced by rmMPO-specific IgG. Recombinant mMPO-specific IgG was adsorbed by incubating with aggregated rmMPO protein, and titre of the resultant supernatant was determined by ELISA. Figure 4A shows the anti-rmMPO reactivity before and after incubation with aggregated rmMPO. The titre in adsorbed antibody decreased to approximately half of the non-adsorbed control. The degree of up-regulation of ICAM-1 expression was also decreased in the cells treated by the adsorbed antibody as compared with the original anti-rmMPO IgG (Figure 4B). Therefore, the increased expression of ICAM-1 seemed to be mediated by the rmMPO-specific IgG molecules and the molecular specificity of the antibody is one of the important factors in the activation.

To investigate the importance of molecular specificity, anti-rmMPO IgG was cleaved into $F(ab')_2$ and Fc fragments by incubating with pepsin (Figure 5A) and the $F(ab')_2$ portion was tested for its ability to up-regulate ICAM-1 expression. As shown in Figure 5B, since there was a significant decrease in the activity of the antibody even after the incubation in sodium acetate buffer without pepsin, the concentration of antibody tested was increased up to 1000 μg/ml. The loss of activity seemed to be due to low-pH-induced denaturation of IgG molecules in the process of digestion in sodium acetate buffer (pH 4.5). The $F(ab')_2$ portion of anti-rmMPO IgG induced a significant increase in ICAM-1 expression in a dose-dependent manner, although it had less activity than the antibody incubated in acetate buffer

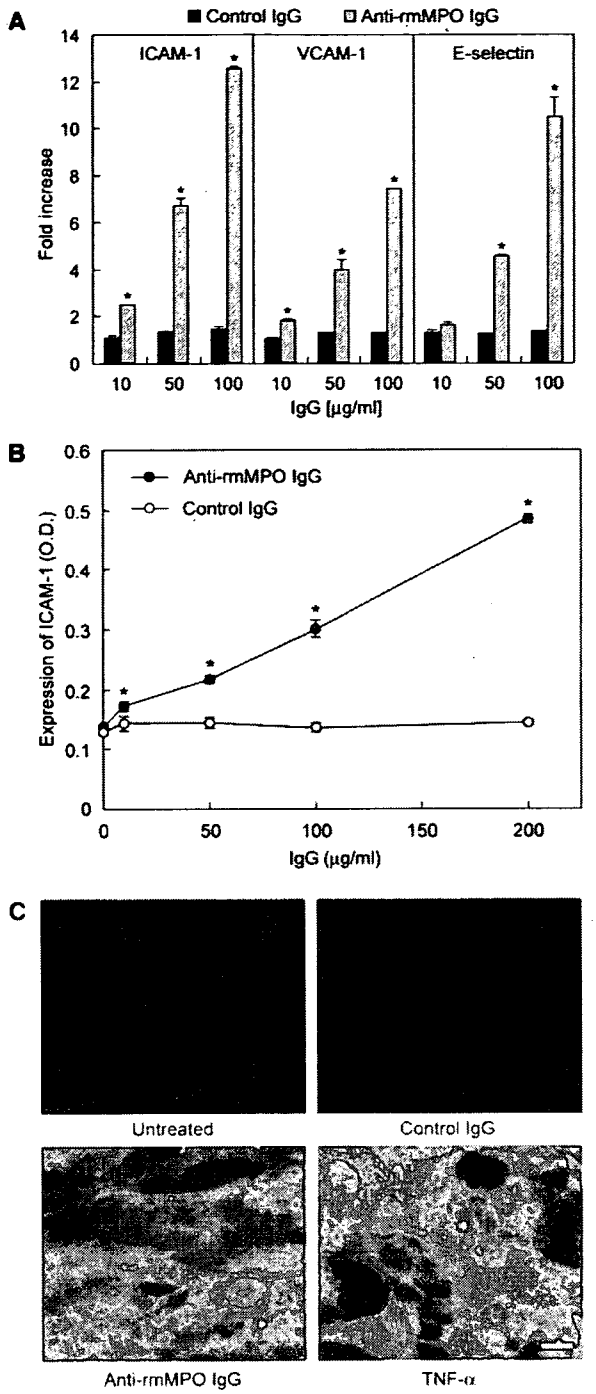

Fig. 2. Up-regulation of adhesion molecule expression in mGEC induced by anti-rmMPO IgG. (**A**) Total RNA was extracted from the mGEC treated with either anti-rmMPO IgG or control rabbit IgG for 4 h. These RNA samples were subjected to real-time RT-PCR analysis as described in Materials and methods. The mRNA levels were normalized by that of non-treated control cells and shown as fold increase. $^*P < 0.05$ *vs* control IgG at same concentration. (**B**) The mGEC were incubated for 6 h with either anti-rmMPO IgG or control rabbit IgG with increasing concentrations. The cells were fixed and ICAM-1 expression level was evaluated by cell ELISA. Data are expressed by optical density at 405 nm. $^*P < 0.05$ *vs* control IgG at same concentration. (**C**) The mGEC were treated with either 100 μg/ml anti-rmMPO or control IgG and then immunostained for ICAM-1 as described in Materials and methods. TNF-α-treated cells served as a positive control. Bar = 20 μm.

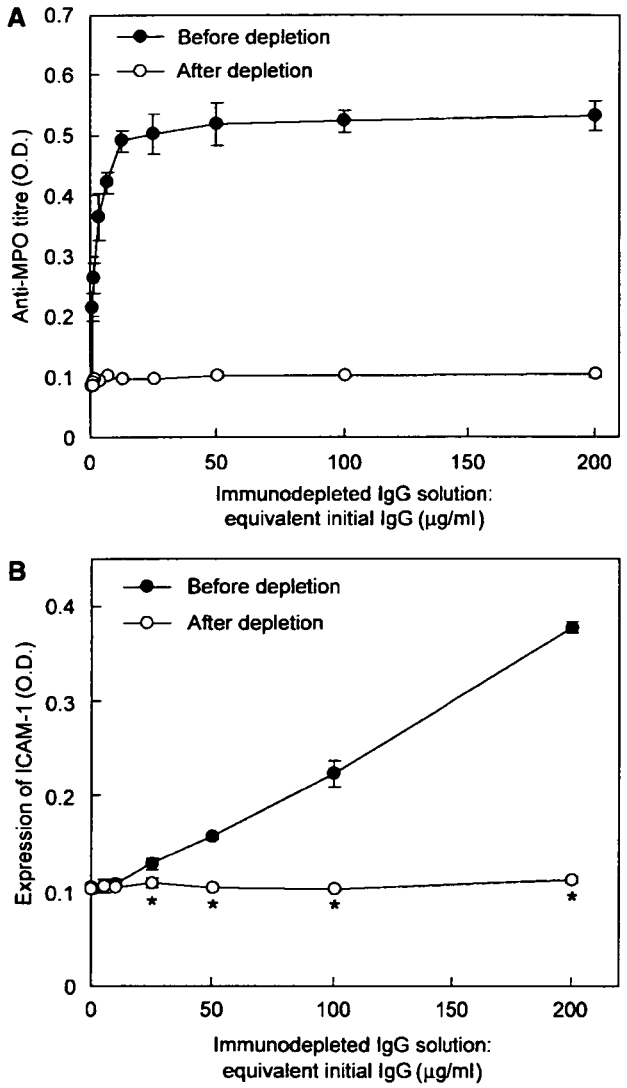

Fig. 3. Up-regulation of ICAM-1 is induced only by IgG molecules and is not attributable to contaminated substances. Anti-rmMPO IgG was incubated with or without protein A sepharose for 1 h at 4°C. After incubation, sepharose beads were spun down and the supernatants were used for the experiments. (**A**) Anti-rmMPO titre of the supernatants was evaluated by ELISA. The anti-rmMPO titre was expressed by optical density at 405 nm. (**B**) mGEC were incubated with the supernatants for 6 h and ICAM-1 expression levels of the mGEC were measured by cell ELISA. Data are expressed by optical density at 405 nm. $^*P < 0.05$ *vs* IgG before depletion at the same concentration.

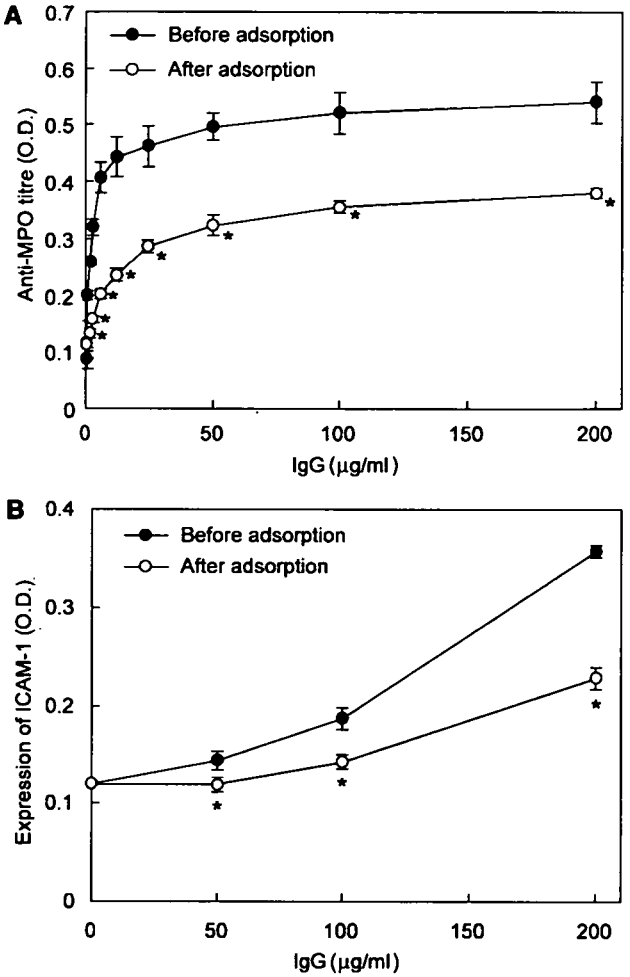

Fig. 4. Antibodies specific to rmMPO induce endothelial activation. MPO-specific IgG was adsorbed by incubating with aggregated rmMPO protein by batch method-like procedure as described in Materials and methods. Protein concentration of the adsorbed IgG was measured and then the IgG was used to measure antibody titre (**A**) and inducibility of ICAM-1 up-regulation (**B**). Both anti-rmMPO titres and ICAM-1 expression levels are expressed by optical density at 405 nm. $^*P < 0.05$ *vs* IgG before adsorption at the same concentration.

without pepsin. The results demonstrate that the antigen specificity of the $F(ab')_2$ portion of anti-rmMPO IgG mediates the enhanced ICAM-1 expression in mGEC.

It has been reported that anti-endothelial cell antibodies from scleroderma or Behçet's disease patients induced adhesion molecule expression [20,21]. To check whether anti-endothelial activity of the anti-rmMPO IgG mediates the activation of the mGEC, anti-endothelial cell activity of anti-rmMPO IgG was compared with control normal rabbit IgG. As shown in Figure 6A, both anti-rmMPO and control IgG showed slight dose-dependent increases in binding to mGEC, which seemed to result mostly from non-specific binding of the antibodies. Contrary to ICAM-1 expression, the difference in the binding activity between control and anti-rmMPO antibodies was undetectable by ELISA. To examine whether antibody binding is specific for living cells, mGEC were treated with anti-rmMPO IgG for 4 h before fixation and bound anti-rmMPO IgG was quantified. As a result, the live mGEC showed binding kinetics similar to fixed mGEC (data not shown). Considering that antigen specificity of anti-rmMPO IgG was necessary to induce the enhanced ICAM-1 expression, a target molecule must be present in mGEC. To test whether there is a target antigen for anti-rmMPO IgG in mGEC, an immunoprecipitation study was performed with anti-rmMPO and control IgGs. As shown in Figure 6B, there were several bands on SYPRO Ruby stain of endothelial proteins specifically

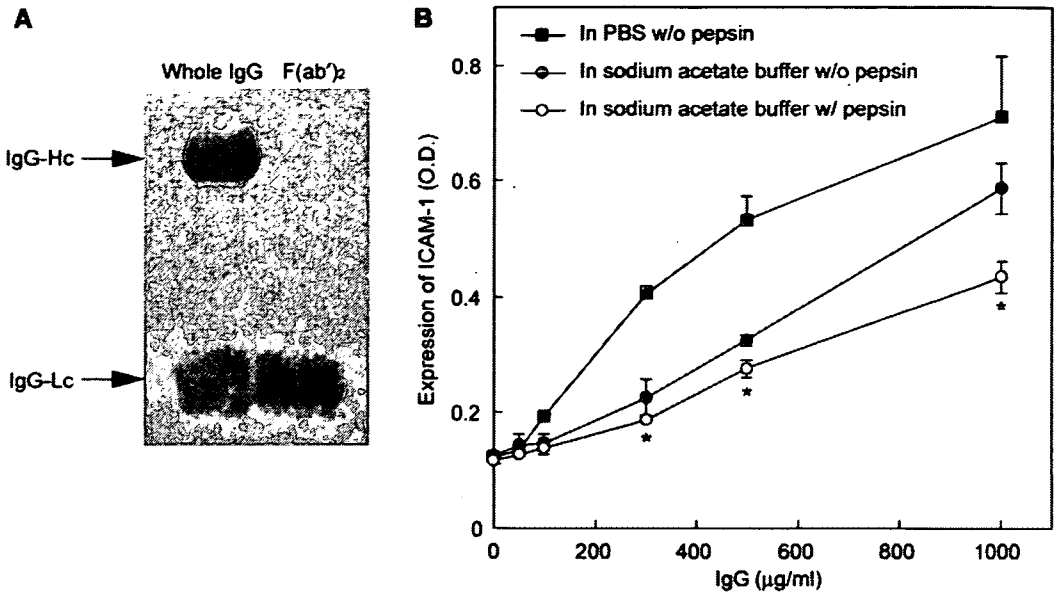

Fig. 5. Up-regulation of ICAM-1 expression in mGEC is associated with antigen specificity of $F(ab')_2$ portion of anti-rmMPO IgG. Anti-rmMPO IgG was digested into Fc and $F(ab')_2$ portions by incubating with pepsin in sodium acetate buffer. (**A**) The original IgG and the isolated $F(ab')_2$ were subjected to SDS-PAGE and stained with Coomassie Blue. (**B**) The $F(ab')_2$ fragments of anti-rmMPO were tested for the inducibility of enhanced ICAM-1 expression. For positive control, antibodies prepared by the same procedure except for incubation without pepsin were included in this experiment. Original anti-rmMPO incubated in PBS without pepsin was also prepared to check the effect of sodium acetate buffer. The ICAM-1 expression levels are expressed by optical density at 405 nm. $^*P < 0.05$ *vs* IgG incubated in sodium acetate buffer without pepsin at the same concentration.

immunoprecipitated with anti-rmMPO IgG. The molecular weights of these proteins did not correspond to those of precursor (90 kDa), heavy chain (57.5 kDa) or light chain (14 kDa) of MPO.

Up-regulation of TNF-α expression by anti-rmMPO IgG and its relation to adhesion molecule expression

In activated endothelial cells, a wide variety of cytokine genes are expressed other than adhesion molecules [27,28]. Inflammatory cytokines are the most important molecules expressed and secreted from the activated endothelial cells. Especially TNF-α is one such cytokine which contributes to the development of glomerulonephritis [13,24,29]. To examine whether the expression of TNF-α is involved in the activation of mGEC by anti-rmMPO IgG, TNF-α mRNA was measured by semi-quantitative RT–PCR. As shown in Figure 7A, the cells incubated for 6 h with various concentrations of anti-rmMPO IgG exhibited an increase in mRNA expression of TNF-α in a dose-dependent manner, whereas the control rabbit IgG failed to do so. Since TNF-α is one of the most potent endothelial activators and induces expression of adhesion molecules including ICAM-1, we examined the effect of neutralizing anti-TNF-α antibody on the enhanced expression of ICAM-1 in mGEC. The mGEC were treated with 100 μg/ml anti-rmMPO IgG in the presence of 0–20 μg/ml neutralizing anti-TNF-α antibody and the expression of ICAM-1 was evaluated. As shown in Figure 7B, there was no inhibition of the enhanced expression of ICAM-1 after 6 h. However, the endothelial activation was partially suppressed by neutralizing anti-TNF-α antibody in a dose-dependent manner if the cells were treated for 18 h.

Discussion

We have demonstrated that anti-rmMPO IgG induced an up-regulation of adhesion molecules. Johnson *et al.* [17] reported an enhanced expression of ICAM-1 in HUVEC with sera or purified IgG from patients with autoimmune vasculitis. However, the relationship between antigen recognition of the antibodies and the up-regulation of ICAM-1 has not been clear. In the present study, activating effects of 'MPO-specific' antibody on endothelial cells have been demonstrated. Since the $F(ab')_2$ fragment of anti-rmMPO IgG could also up-regulate the adhesion molecule, it was concluded that antigen recognition was involved in this process. However, we were unable to demonstrate whether cross-linking of the target antigen was involved because digestion and purification of Fab fragment led to loss of activity.

It has been reported earlier that MPO is not expressed in HUVEC [30] and we also confirmed by RT–PCR analysis that the isolated mGEC also do not express murine MPO (data not shown). The difference

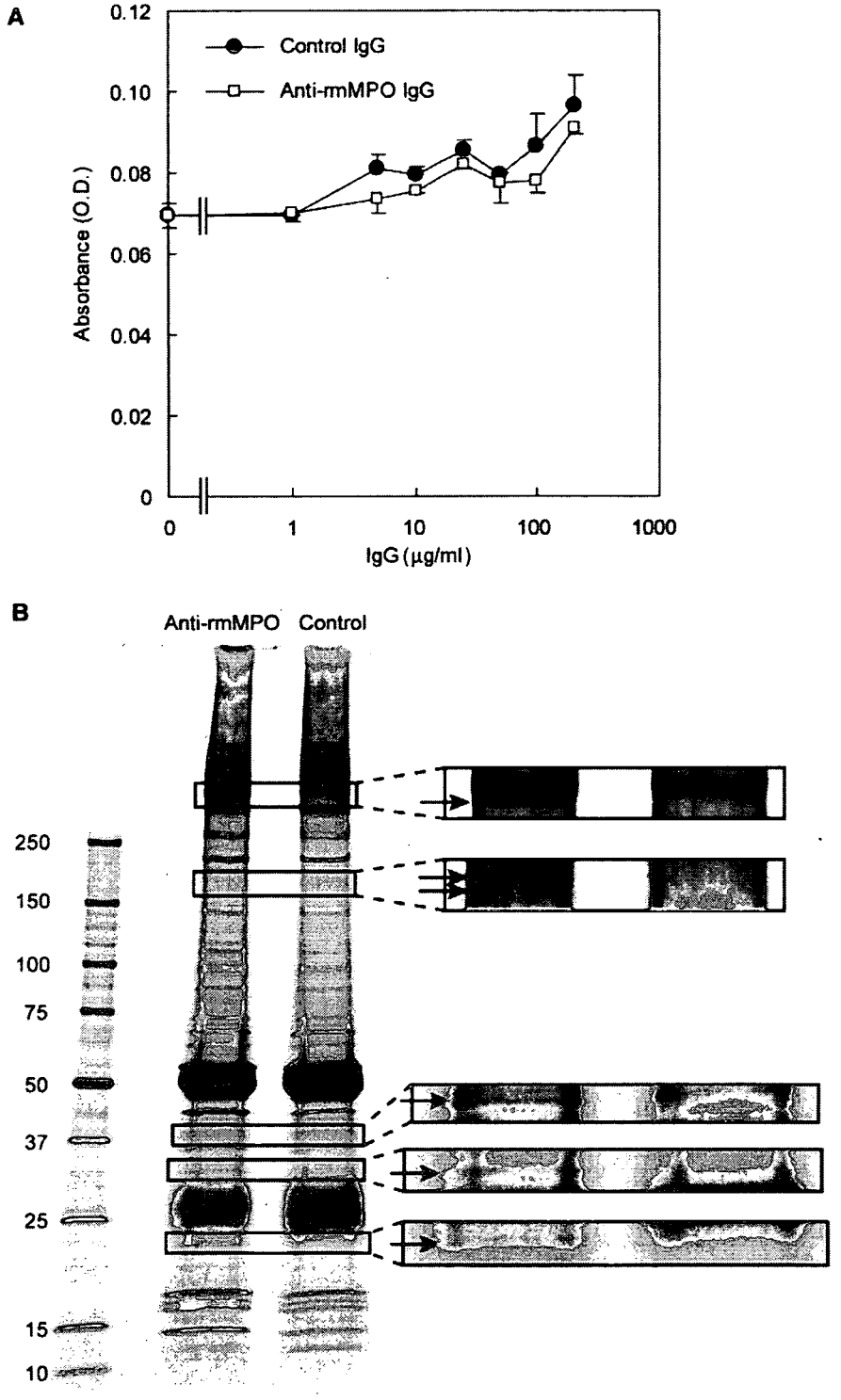

Fig. 6. Anti-endothelial activity of anti-rmMPO IgG. **(A)** Binding of anti-rmMPO IgG to fixed mGEC was evaluated by cell ELISA as described in Materials and methods. The anti-endothelial activities are expressed by optical density at 405 nm. **(B)** Immunoprecipitates from whole-cell lysate of mGEC either with anti-rmMPO or with control IgG were analysed by SDS-PAGE and SYPRO Ruby staining. The anti-rmMPO IgG-specific bands are magnified.

in anti-endothelial activity between anti-rmMPO and control rabbit IgG was undetectable by ELISA, presumably because non-specific binding (e.g. binding to Fc receptors) was dominant. However, since we successfully detected several bands on SYPRO Ruby stain of immunoprecipitates specific for anti-rmMPO IgG, we believe that only subtle binding of anti-rmMPO IgG to a certain molecule existing

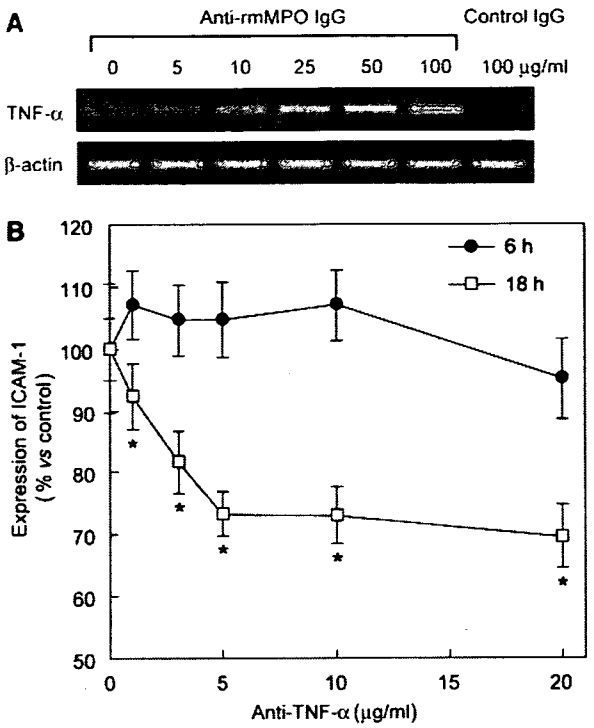

Fig. 7. Involvement of TNF-α expression in up-regulation of ICAM-1. (**A**) The mGEC were incubated with indicated concentrations of anti-rmMPO IgG or control rabbit IgG for 6 h and then mRNA expression of TNF-α was determined by semi-quantitative RT–PCR method. RT–PCR products of β-actin of the same samples were included as internal control. (**B**) Inhibition of up-regulation of ICAM-1 expression by neutralizing anti-TNF-α antibody. The mGEC were incubated for 6 or 18 h with 100 μg/ml anti-rmMPO IgG in the presence of the indicated concentrations of neutralizing anti-TNF-α antibodies. ICAM-1 expression levels of the cells were measured by cell ELISA and were normalized by that of the cells treated with 100 μg/ml anti-rmMPO IgG in the absence of neutralizing antibody. $^*P < 0.05$ *vs* cells treated with anti-rmMPO IgG in the absence of neutralizing antibody.

on mGEC can trigger the activation of mGEC. Therefore, we speculate that certain molecules expressed in mGEC (probably on plasma membrane) may share the epitope of anti-rmMPO IgG and transduce signals leading to the enhanced expressions of adhesion molecules. Identification of the molecules bound with anti-rmMPO IgG will be analysed in a future study.

Although it has been reported earlier that monoclonal antibodies against MPO do not induce endothelial activation [17], yet the present study shows its induction of adhesion molecules. We, however, here utilized the polyclonal anti-rmMPO IgG containing antibodies with many different binding sites (epitopes) to MPO. The reports, on epitope mapping of MPO-ANCA using recombinant deletion mutants of MPO, have demonstrated that MPO-ANCA in sera of the patients recognizes different epitope sites of MPO with a restricted number of epitopes located on heavy chain of MPO [31–33]. Furthermore, the epitope recognition profiles are also related to clinical features. In other words, only a few clones targeting the risk epitopes can initiate and progress MPO-ANCA-associated glomerulonephritis. Therefore, the polyclonal antibody used in this study seems to contain the antibodies against the specific risk epitopes. An epitope mapping of the anti-rmMPO IgG which activated mGEC in the present study may help to elucidate the cross-reactivity of anti-rmMPO IgG and the mechanism of endothelial activation related to pathogenicity of MPO-ANCA.

The results of the neutralizing anti-TNF-α antibody experiment show that although TNF-α was not likely to be a primary activator in the initial period (6 h), endothelial ICAM-1 expression was enhanced by TNF-α secreted from activated mGEC after 18 h. The production of TNF-α induced by anti-rmMPO IgG may in part contribute to the up-regulation of adhesion molecules in an autocrine fashion. However, it still remains controversial whether anti-rmMPO IgG directly induces the up-regulation of adhesion molecules because the other inflammatory mediators might be released from mGEC, and subsequently activate mGEC even just after the binding of anti-rmMPO IgG. Besides TNF-α, a wide variety of inflammatory cytokines or chemokines such as interleukin (IL)-1, IL-8 and monocyte chemoattractant protein-1 (MCP-1) are released from endothelial cells in response to stimuli [34,35]. These cytokines/chemokines activate endothelial cells and up-regulate ICAM-1 expression [36–38] and have been further reported to correlate with clinical presentation of MPO-ANCA-associated glomerulonephritis. Adhesion molecules, investigated in this study as well as TNF-α are mainly expressed through NF-κB activation [39–42]. Therefore, it is expected that anti-rmMPO IgG activated NF-κB which subsequently induced up-regulation of adhesion molecules and inflammatory cytokines/chemokines. Blocking antibodies to these cytokines/chemokines or inhibitor of NF-κB may further reveal the role of these mediators in up-regulation of adhesion molecules.

Sensitivity of endothelial cells to cytokine exposure or injury mediated by activated neutrophils differs with their origin [22,43]. We also examined the change in ICAM-1 expression by anti-rmMPO IgG using other microvascular endothelial cells such as primary mouse lung endothelial cells (mLEC) and pancreatic islet endothelial cell line (MS1). These cells also showed an increased mRNA expression of ICAM-1 (see 'Supplementary' data), indicating that these effects were commonly seen in microvascular endothelium and not limited in the kidney. MPO-ANCA is believed to be associated with the development of small vessel vasculitis. Therefore, the comparison of adhesion molecule expression by anti-rmMPO IgG between endothelial cells from small and large vessels might help to understand the pathogenesis of ANCA-associated vasculitis.

In conclusion, the present results clearly indicate that the anti-MPO antibody up-regulates the expression of ICAM-1, VCAM-1 and E-selectin in mGEC,

suggesting that not only neutrophils but also glomerular endothelial cells are activated by MPO-ANCA and contribute to neutrophil adhesion to GEC, thereby increasing glomerular neutrophil infiltration in initiation and progression of pauci-immune glomerulonephritis. It is still unknown how anti-rmMPO IgG stimulates the expression of adhesion molecules in mGEC, although our study revealed that antigen recognition of anti-rmMPO IgG is necessary for the stimulation. Future *in vitro* studies on the aforementioned issues will provide a more precise mechanism for endothelial activation in pauci-immune glomerulonephritis. Furthermore, using the animal model of MPO-ANCA-associated glomerulonephritis [44], we will investigate the role of MPO-ANCA-induced expression of adhesion molecules and inflammatory cytokines/chemokines in mGEC *in vivo*.

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Conflict of interest statement. None declared.

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血管炎の病態と好中球

鈴木和男

〔SUMMARY〕 好中球は，活性酸素産生やmyeloperoxidase(MPO)が産生するOCl^-によって感染防御にはたらく．しかし，血管炎患者血清中に上昇する自己抗体MPO-ANCAおよびMPOが発症に関与していることがMPOノックアウトマウスによって証明された．また，MPOの重鎖のNおよびC末端にエピトープをもつMPO-ANCAが病態の重症化と関連することが判明した．一方，急性進行性糸球体腎炎(RPGN)を自然発症する病態モデルSCG/Kjマウスや真菌分子*C. albicans* extract(CADS)，*C. albicans* water soluble glycoprotein(CAWS)誘導の冠状動脈炎を誘発するマウスの解析から治療方法が検討されている．最近，MPO抗体が糸球体内皮細胞に直接反応してICAM-1発現を誘発することがわかったことから，*in vivo*でのMPO抗体の役割を知るために量子ドットを使ったトレーザ解析法が有用となってきている．〔臨床検査　51：1071-1080, 2007〕

〔KEYWORDS〕 anti-neutrophil cytoplasmic antibody(ANCA)関連血管炎，活性化好中球機能，myeloperoxidase，血管炎モデルマウス，MPOノックアウトマウス

MPOを抗原とする自己抗体MPO-ANCA関連血管炎

1. MPOおよびMPO自己抗体による血管炎発症への関与

MPOと好中球の動態が，慢性炎症疾患に関与していることが注目されている．血管炎患者には血清中に好中球自己抗体(anti-neutrophil cytoplasmic antibody；ANCA)が上昇することや，ANCAと好中球が血管炎の病態に関与していることが明らかになってきており，好中球はその抗体ANCAとともに血管炎に関与していることが容易に理解できるようになってきた．血管炎の患者の血中には，高いMPO活性とともに，活性化された好中球が循環していることが明らかになっている[1]．その結果，循環している好中球の活性化が血管炎の発症と病態の進行に関与していると推定されている．また，自己抗体MPO-ANCA抗体による反応には，FabやFcγレセプターが関与して好中球を活性化しているのではないかと推定されている．このように，その抗体MPO-ANCAが生体側に不利な細胞障害を引き起こす好中球の活性化にかかわっていることが明らかにされてきている．最近のわれわれの研究からMPO-ANCAが直接血管内皮細胞に作用することも報告した．

2. MPO-ANCAの血清レベルが血管炎のマーカーとして利用されている

MPO-ANCAは，血管炎を呈する疾患にその上昇が認められることから，臨床マーカーとして利用されている．腎炎をはじめ血管炎や川崎病や関節リウマチや全身性エリテマトーデス(systemic lupus erythematosus；SLE)においても，MPO-ANCAが高値を示すケースがある．また，われわれが証明したMPO-ANCAの抗原であるMPO[2]もMPO-ANCAと呼応して血管炎の発症に関与する．

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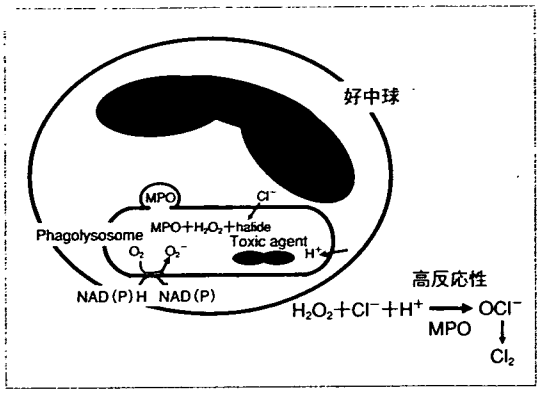

図1 MPOはOCl^-を産生—ファゴゾーム中での産生機構

3. 好中球活性化によってさらにMPO-H_2O_2系が作動して炎症局所での血管傷害を進展させる

本来は殺菌に作動する好中球のMPO-H_2O_2系が条件・状況によって生体側に不利な細胞に障害を引き起こし炎症を惹起する．本来好中球は，感染部位へ到達して貪食して食胞をつくるやいなや，好中球内の顆粒(ライソゾームを含む4種の顆粒)と融合してファゴライソゾームを形成し，MPOをはじめ顆粒内の消化・不活化酵素をそのファゴライソゾームの中に放出する．これと同時に，活性酸素も産生し，殺細菌，殺ウイルス・殺真菌をする．活性酸素産生酵素NADPH(nicotinamide adenine dinucleotide phosphate)oxidaseに加え，MPO酵素も，殺菌能に不可欠である．NADPH oxidaseによって産生されたO_2^-とプロトンとCl^-によって次亜塩素酸を造る．すなわち，MPOは，NaClとH_2O_2プロトンを基質としてOCl^-を産生する(図1)．

実際，感染によってこのMPOが血液中に放出され，炎症がおさまると同時にクリアされCRPと同様の血中レベルの変動を示す．しかし，腎炎患者や川崎病患児の好中球の機能の解析から，血管炎の患者の血中には高MPO活性とともに活性化好中球が循環し，MPO-ANCAとともに体側に不利な細胞障害を引き起こすことが明らかにされてきている．この活性化好中球は，続発する炎症や感染によって血管障害の誘発に関与すると推定されている．Fcγレセプター欠損マウスでは，腎炎が発症せず，好中球の活性化が関与していることが示されている．

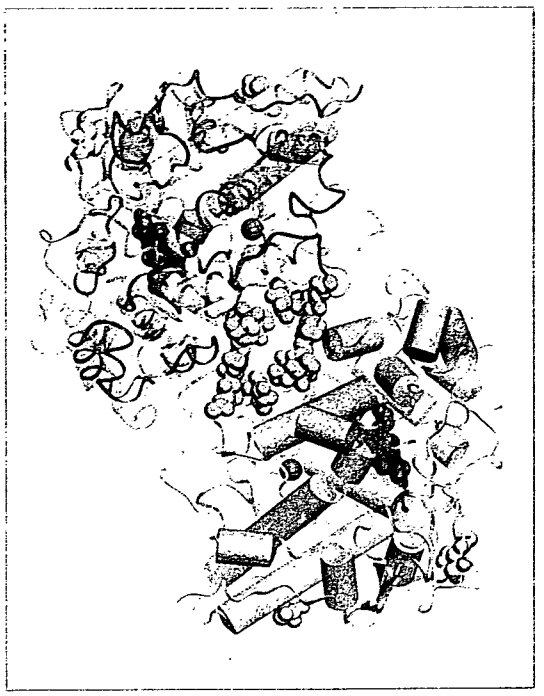

図2 MPOの分子構造は，短鎖(14 kDa)と長鎖(59 kDa)の2分子にヘム2分子をもつ158 kDaのヘテロテトラマーの糖蛋白質

MPO-ANCA関連血管炎の病因解析

しかしながら，血管炎疾患患者血清中のMPO-ANCA抗体価の変動は，必ずしも疾患の病態と連動していないことが臨床面から指摘されており，病態と密接に関与するMPO-ANCAについて検討することが重要になっていた．このため，筆者らは，血管炎の病態を判定するためのMPOの分子構造に基づいてdeletion mutantを用いたMPO-ANCAエピトープ解析用パネルを作製した[3]．

1. MPO分子の構造

MPOの分子構造は，light chain (14 kDa)とheavy chain(59 kDa)の2分子にヘム2分子をもつ158 kDaのヘテロテトラマーの糖蛋白質で，等電点10.3以上で，第17染色体にコードされている(図2)．MPOの構造不全は，遺伝子の異常のみならず遺伝子発現調節部位で調節している転

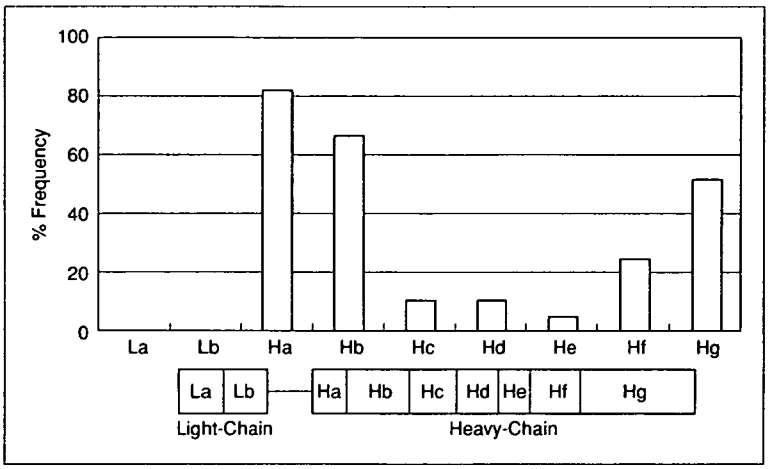

図3　重症化と関連するMPO-ANCA抗体のエピトープ

写因子の機能不全により異常をもたらされる．

2. MPO-ANCA抗体のエピトープ(MPO分子との反応部位)による病態との関係を解析

MPOを7部分に分けて，*E. coli*を用いたリコンビナントフラグメントからなるパネルセットを作製した．ウエスタンブロット法やELISA(enzyme-linked immunosorbent assay)によるエピトープ解析から，特定の反応部位が病態と関連することを認めた[4]．その結果，MPOのL鎖とは全く反応せず，主としてH鎖のNおよびC末端に単独で反応するエピトープをもつMPO-ANCA抗体が重症化と関連していた(**図3**)．この解析結果は，特定のモノクローナル抗体が重症化と関連していると予想された．さらに，「厚生省・難病血管炎班(橋本博史班長)」において，種々の血管炎患者のMPO-ANCA陽性を認めた176例の血管炎患者血清におけるMPO-ANCAのエピトープを解析した．約70%の血管炎患者の血清は，エピトープを示した．特に，多発動脈炎(polyarteritis nodosa；PN)および顕微鏡的多発動脈炎(microscopic polyarteritis；MPA)患者の血清は，MPOのH鎖のNおよびC末端に単独で反応するエピトープをもつモノクローナル抗体であり，他の血管炎の血清MPO-ANCAは，末端ではない部位のエピトープ，2つ以上，すべてに反応するエピトープを示した．以上から，抗体のクロナリティは，血管炎疾患および病態と関連があることを示唆している．

エピトープ解析からMPO-ANCAのモノクローナル抗体が血管炎の進行とかかわることがわかった．また，MPO-ANCAは，活性化した好中球の表面に出てくるMPOをFabにより認識し，Fcγレセプター欠損マウスでは腎炎が発症しないことから，好中球の活性化に関与する抗体および免疫複合体は特異性が高いモノクローナル抗体であることが明らかになってきている．一方，川崎病の治療に用いられている免疫グロブリン製剤は，MPO-ANCAを含んでいるが，ポリクローナルであることを確認している．健常者血中にもMPO-ANCAが含まれているのと同様である．これらのことから，病因性の高いモノクローナル抗体が，ポリクローナルによって弱められていることが治療に有効性を示す一因である可能性もある．これらの結果は，治療による病態の評価基準作成や血管炎の重症度の評価に重要であると考えられる．このエピトープ解析から，抗体による治療あるいは，大量免疫グロブリン治療が有効であることも考えられていた．国内においては，大量免疫グロブリンの予備的治療が行われ，報告された[5]．

3. 病因解析のための血管炎モデルマウス

1) 進行性糸球体腎炎(rapidly progressive glomerulonephritis；RPGN)-SCG/Kjマウス

モデルマウスを用いた血管炎の病因の解析や治療に関する研究が進んできており，腎炎や血管炎を自然発症するマウスには，NZB/WF1，MRL/lpr，SCG/Kjがある．なかでも，SCG/Kjマウスは，半月体形成性糸球体腎炎を高率かつ急速に自

表1 MPO欠損マウスにおける冠状動脈炎の減少とMPO-ANCA産生の抑制

Mouse	N	MPO-ANCA (hEU/ml)	anti-ds-DNA antibody (IU/ml)	Coronary arteritis incidence (%)
CADS injection				
C57BL/6	4	11.1±6.0	37.1±33.5	100
MPO-deficient	5	4.8±2.2	22.3±3.8	40
PBS(-)injection				
C57BL/6	10	3.5±4.5	23.4±8.4	0

然発症し，その病態の発症・進行においては，好中球機能およびMPO-ANCAが関与している．SCG/Kjマウスは，リンパ節腫脹が観察され，MPO-ANCAが腎炎の発症とともに増加して半月体形成を伴う腎炎を早期に自然発症する[6]．腎炎進行度を3段階に分類：20週齢までのSCG/Kjマウスについて，尿蛋白質量から，腎炎進行度を3段階に分類できる．発症前期段階：30 mg/dl以下，初期段階：3～300 mg/dlおよび後期段階：300 mg/dl以上．この分類により，末梢好中球数，好中球機能，血清MPO-ANCA値および腎臓への好中球の浸潤度を調べた[4]．腎炎の発症・進行の時期の3段階のうち，初期段階において，無刺激の好中球からのMPO放出が高値を示す．しかし，細菌由来走化性ペプチドfMet-Leu-Phe誘導のMPO放出では，腎炎の3段階すべての時期を通じて亢進しており，時期間の差が見られなかった．血清中のMPO-ANCA値は，初期段階から高値を示した．また，糸球体への好中球浸潤とも相関があり，腎臓障害スコアおよび半月体形成スコアとも弱いながらも相関が認められている．初期段階において，無刺激MPO放出が，糸球体への好中球浸潤，腎臓障害スコアおよび半月体形成スコアと有意に相関しており，腎炎発症と進行には，活性化好中球が関与していることが示唆される．以上から，糸球体腎炎の発症SCG/Kjマウスでは，末梢血中に活性化好中球数が増加し，炎症を拡大し，腎臓への活性化好中球浸潤が半月体形成に重大な影響を及ぼしていると推定される．また，好中球が放出するリスク分子により正常細胞破壊やconnective tissue破壊が起こる．このように，活性化好中球によって腎臓障害が進行するものと推定される．SCG/Kjマウスは，急性進行性腎炎の発症機構を明らかにするうえで重要である．さらに，RPGNの治療モデルとして利用でき，治療法の開発に有用であると思われる．

RPGNの発症・進行には，好中球活性化因子がキーになると思われる．しかし，好中球の活性化因子は特定されていない状況である．マウスのモデルから得られた因子をヒトの血管炎・腎炎発症機序の解析や治療法に向けた利用が重要であろう．最近，Hamanoら[7]によりSCG/Kjマウスを用いたMPO-ANCA産生にかかわる因子群の遺伝子座領域を染色体マップし，*Man-1*と名づけた．また，BSA(bovine serum albumin)誘導の半月体形成腎炎モデルも報告されている[8]．

2）冠状動脈炎：真菌分子が誘発する血管炎マウス

冠状動脈炎を誘発するモデルマウスがある．その1つに，*Candida albicans* extract(CADS)誘発の冠状動脈炎マウスである．このマウスは，血管炎発症とともに，血中にMPO-ANCAが上昇する．MPO遺伝子欠損マウスでは，血管炎の発症とともにMPO-ANCAが低下することから，MPOが主たる抗原になって，血管炎の発症に関与していることが明らかになっている[9]．CADS誘導の冠状動脈血管炎マウスについて，野生型マウス(C57BL/6)において，冠状動脈血管炎発症率とMPO-ANCA値に，正の相関が認められたことから，CADS抽出物誘導の冠状動脈血管炎の発症に，MPO-ANCA産生の関与が示唆された．冠状動脈血管炎発症へのMPOの関与を解析するため，MPO-KOマウスに冠状動脈血管炎を誘導させた．MPO-KOマウスにおいて，CADS誘導の血管炎発症率と血清中のMPO-ANCA値を測定し，野生型のそれと比較した．その結果，MPO-KO群の血管炎の発生率は40%となり，対照群100%より激減した．一方，血清中のMPO-ANCA値は，MPO-KOマウスでは，野生型マウ

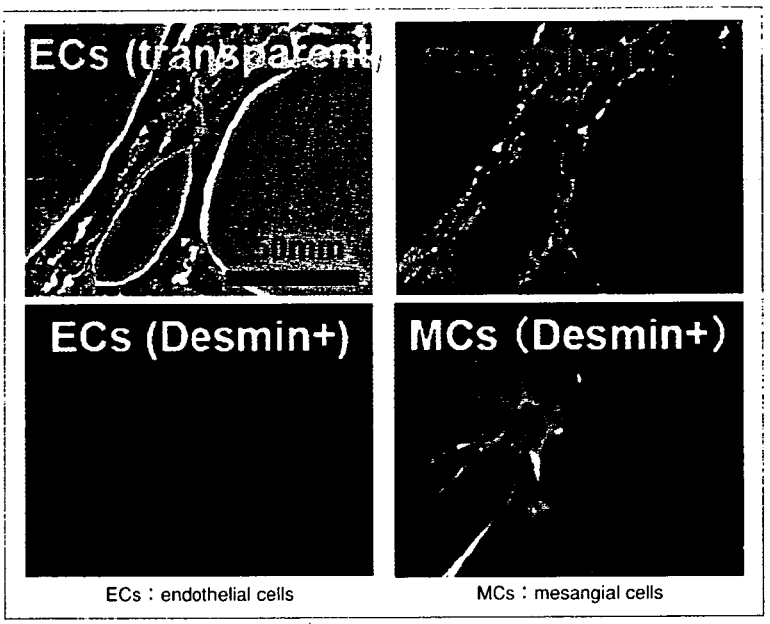

図4　マウス糸球体内皮細胞の初代培養細胞

スに比べ，ほぼPBS投与に匹敵するほど低下していた(表1)．最近，さらに高率に血管炎を誘発する *Candida albicans* water soluble glycoprotein (CAWS)冠状動脈炎をマウスに誘発することが明らかにされ，その発症初期過程に好中球の活性化が関与している[10]．

4. 血管内皮細胞に直接作用するMPO-ANCA

血管炎の発症の要因には活性化した好中球が関与していると推定される一方，血管内皮細胞にも直接影響を与えている可能性が示唆されている．そこで，筆者らは，MPO抗体による血管炎誘導初期の血管内皮細胞障害について解析した．マウス糸球体から得た血管内皮細胞の初代培養(図4)を用いて，活性化好中球の糸球体内皮細胞へのMPO抗体の作用を解析した．anti-mMPO抗体は，糸球体内皮細胞のICAM-1，VCAM-1，E-selectinの発現を誘発し(図5)，ICAM-1のmRNAの発現の上昇が認められた．MPO-ANCAによる内皮細胞障害に関与するサイトカイン18種類を定量した結果，anti-mMPOは，糸球体内皮細胞のKCケモカインの産生の顕著な増加が認められた．以上から，血管炎の発症は，MPO-ANCAが，好中球への作用に加え，直接血管内皮細胞にも結合してICAM-1の発現を上昇させ，KCケモカインを放出し，活性化した好

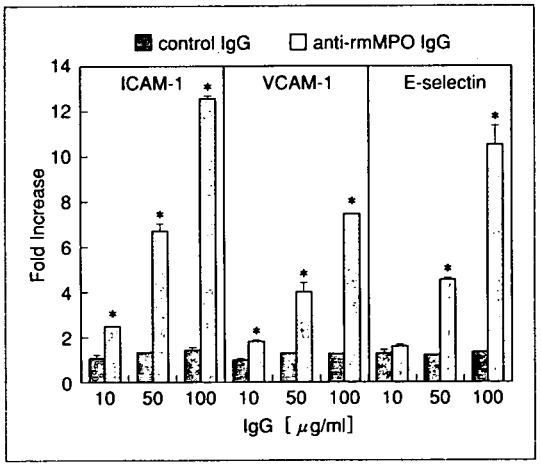

図5　anti-mMPO抗体によるmGECのadhesion moleculesの発現

中球がMPOおよび活性酸素を産生することで，組織にとどまって二次的な血管内皮細胞障害を起こすことが考えられている[11]．

5. 好中球の活性化によって細胞表面に表出するMPO分子とそのトレース

MPO-ANCA抗体は，間接的直接的に好中球の活性化や血管内皮細胞の障害性に関与していることが明らかにされてきているが，MPO-ANCAがどのように生体内で関与しているかは不明である．一方，*in vivo* でのイメージング技術による

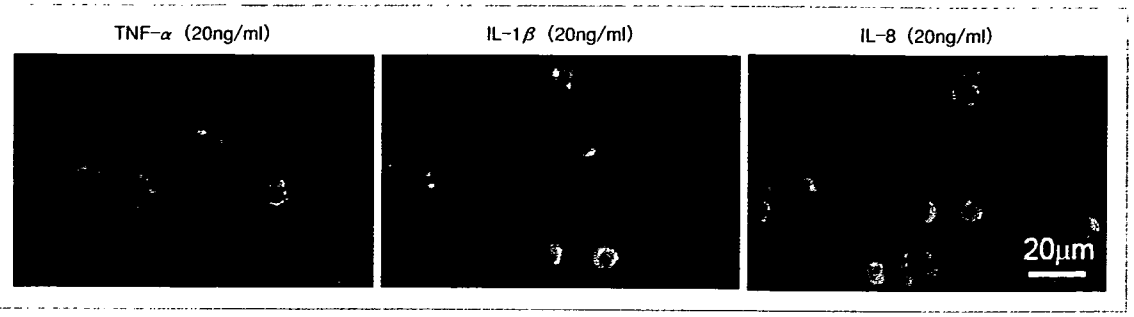

図6　TNF-α，IL-1β およびIL-8刺激による好中球細胞膜へのMPO表出

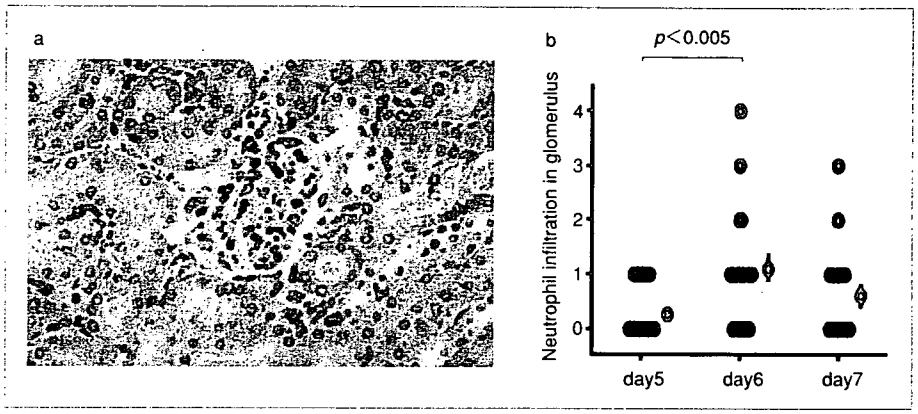

図7　antiMPO-QD抗体およびCAWS投与による糸球体への好中球の浸潤
a：HE像，b：糸球体1個あたりに浸潤した好中球数．

解析には，ナノプローブの利用が必須である．量子ドット(QDots)を用いた血管炎の発症要因の解析が検討されてきた．具体的には，われわれが開発した血管炎モデルマウスに，MPO抗体を投与して血管炎発症過程をしらべ，糸球体から単離したプライマリー培養の糸球体血管内皮細胞にQDで標識したMPO抗体(QD-antiMPO)を加え，*in vitro*でのQD-antiMPOの糸球体血管内皮細胞への結合と作用について解析し，MPO抗体の血管内皮細胞への結合を可視化した．一方，*in vitro*でヒト好中球を好中球の走化因子のfMetLeuPheペプチドで刺激することで，MPOの細胞外への表出が観察され，炎症性サイトカインであるTNF (tumor necrosis factor)-α，IL (interleukin)-1β およびIL-8の刺激によっても同様にMPOの表出が確認された(**図6**)[12]．糸球体への好中球の浸潤度合いを検討し，好中球浸潤は，MPO抗体とCAWSの投与で有意に増加した(**図7**)[12]．

以上から，活性化好中球が細菌感染や炎症性サイトカインの産生に伴って，細胞質顆粒のMPO分子を細胞表面へと表出することが判明した．また，MPO-ANCAが活性化好中球の表面に表出したMPOに反応していることが示唆された．したがって，活性化好中球の細胞表面に出たMPOとMPO抗体が結合して，糸球体の機能に影響を与えることが十分考えられる．

6. MPOと好中球機能不全によるその他の疾患—日和見感染の誘発と真菌感染の原因になる好中球機能不全

MPOを欠損した患者の病態や欠損マウスの解析[13]，あるいは，これら遺伝子のノックアウトマウスを用いた*in vivo*の感染実験およびNADPH oxidaseの解析から，ラジカル酸素産生能の低下が真菌感染抵抗性の低下をもたらしていることが解明された．NADPH oxidase欠損は，*Aspergil-*

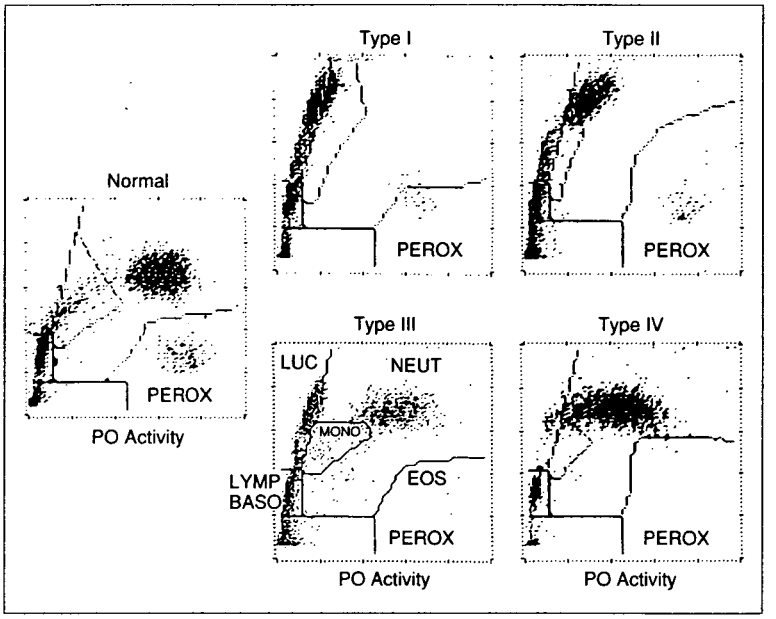

図8　ADVIA シリーズによる MPO 活性の不全

lus 感染を主とした真菌感染を誘発し，CGD となって重篤化すること，および，NADPH oxidase (gp91^{phox}) 欠損マウスの解析から，NADPH oxidase は，*Aspergillus* 感染への抵抗性に極めて重要であることが判明していたが，その下流に位置する H_2O_2 も産生されないことから CGD は，O_2^- と OCl^- の両者の産生不全が原因となって重篤になると思われる．事実，MPO と NADPH oxidase (構成分子 gp91^{phox}) それぞれのノックアウトおよびこれらのダブルノックアウトマウスの解析から証明された[14]．真菌はサイズが大きいため好中球は貪食できない場合にも，癌細胞を障害するのと同様に好中球が産生する活性酸素や OCl^- を放出して，真菌表面の分子を変性させて，機能を不活性化するものと推定される．

好中球殺菌酵素 MPO 欠損とそのホモログの研究

MPO は，その語源から myeloid cells (骨髄細胞) に特徴的な peroxidase であり，主として好中球に存在している．末梢血をまわっている単球にもごくわずかに存在している．2000 年に "The peroxidase multigene family of enzymes"[15] というタイトルの本にこれまでの MPO を含む peroxidase の研究がまとめられた．その内容は，ミュンヘンにて開催された第 2 回国際 Peroxidases 会議のもので，それ以後のめざましい進展は網羅されていない．2004 年に京都にて開催された第 4 回会議の Proceedings に最新の情報が網羅されている[16]．また，gp91^{phox} ホモログとして Nox (NADPH oxidase) ファミリーが登場し，2000 年に熱海で開催された第 6 回 MPO 会議で Krause と Sumimoto の研究がはじめて Nox として登場した[3]．その機能は，生体防御，直接には食細胞の NADPH oxidase，間接的には，Nox1 や DUOX1，DUOX2 があり，ホルモン合成とかかわる DUOX がある．2006 年 10 月にはスイスで第 1 回 Gordon Research Conference-Nox が開催されるに至った．

1. 日本人における MPO 欠損頻度

日本人における遺伝的 myeloperoxidase 欠損頻度：臨床血液検査機器 Technicon H シリーズ (現在は，ADVIA シリーズ)，MPO 活性と flow cytometory によって好中球数を算出することに注目し，そのデータ (**図 8**) から得られる MPO Index (MPIX) から欠損型が 4 つに分類されている．本機を導入している施設に型別のデータの協力が得られた施設の回答から MPO 欠損頻度を求められている．有効回答のあった 50 施設のデータの

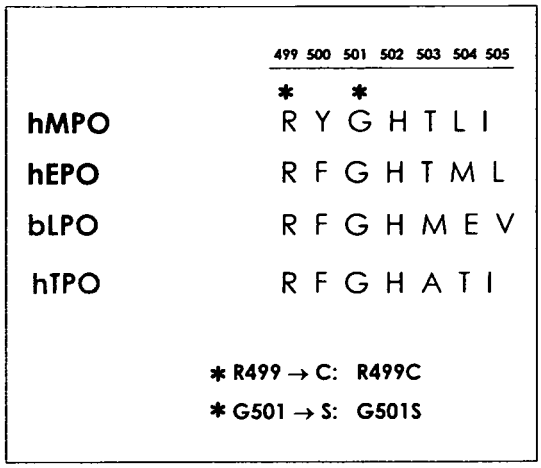

図 9　ヒトの MPO 遺伝子欠損(日本)：G501S および R299C の遺伝子変異

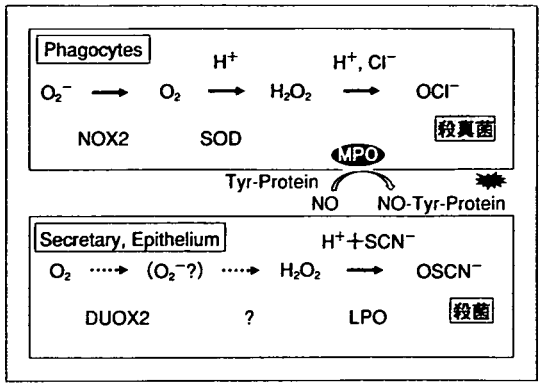

図 10　MPO と LPO の反応と生理的機構の相違

集計より，国内の完全欠損型は 16 施設に 26 人で，完全欠損の発生頻度は施設平均では 1 人/57,135 人である．部分欠損型は 20 施設に 129 人あり，施設平均では 1 人/17,501 人である[17]．一方，欧米の解析からは，イタリアでは，1 人/4,000 人であり，米国では，1 人/2,000～4,000 人と報告されている．また，その原因になっている遺伝子変異は，R569W[18,19]，Y173C[20]，and M251T[21]，G501S[22]が報告されている．日本人の欠損の解析から R299C も存在し，これら 2 例(G501S および R299C)の遺伝子変異は，分子構造上重要な部位(**図 9**)であり，その 2 つの欠損による分子機能についても明らかになった[23]．

2. MPO 欠損マウスの病態

個体の真菌感染防御における MPO の役割はいまだ明確ではないことから，Aratani ら[14]は，MPO-KO マウスを作製して，このマウスの生体防御能の異常を解析している．MPO-KO マウスは野生型マウスに比べて，*C. alibcans* と *A. fumigatus* のいずれに対しても初期感染防御能の著しい低下が認められ，その低下は *C. alibcans* においてより顕著である．これらの MPO-KO マウスの結果は，MPO 欠損症の臨床症状が，*C. alibcans* 易感染傾向が示されている臨床像の報告を証明した結果となっている[21]．

MPO 欠損マウスは，MPO が関与すると思われる機構解析にパワーを発揮する[14,22]が，予想外に atherosclerosis で相当高い値を示すこともあり[24]，ヒトとマウスの違いもある可能性も否定できない．

3. MPO とホモログを示す peroxidase 酵素群

MPO とアミノ酸配列のホモログのある peroxidase に，eosinophil peroxidase (EPO)，lactperoxidase (LPO)，thyroid peroxidase (TPO)，platelet peroxidase (PPO)，dualxidase-1 (DUOX1) がある[9]．このうち，DUOX については，Nox ファミリーに属するが，Ca 結合ドメインの EF-hand をもつが peroxidase 活性はなく，リコンビナントを作製してヘムの結合が確認できていない．それ以外は，peroxidase 活性をもっており，ヘム酵素である．これらの酵素は，基質として H_2O_2 とハロゲン(X)などの 2 つが必要であることが共通点である．X により，産生される産生物に相違があり，その産生物によりターゲットされる細菌などに違いがあると考えられている．前述したように MPO が産生する OCl^- は，真菌の膜成分を破壊し，組織障害を誘発する．一方，Tom Leto ら[25]によれば，LPO が産生する $OSCN^-$ は，分泌組織の上皮細胞上に産生されて，殺菌に有効に作用していると考えられている．両者の役割を図に示した(**図 10**)．MPO と LPO の生体内での比較は，今後，新しい展開が期待される．

まとめ

MPO は，好中球の，殺細菌，殺真菌の際にはたらくが，その自己抗体 MPO-ANCA は，血管炎患者血清中に上昇し発症に関与する．そのエピ

トープと病態との関連についての解析から，H鎖のNおよびC末端に反応するエピトープが重症化と関連していた．治療法開発や発症機構解明には，RPGN自然発症病態モデルにSCG/KjマウスやCADS，CAWS誘導の冠状動脈炎を誘発するマウスが有用となってきている．さらに，MPO抗体は，糸球体内皮細胞のICAM-1発現とKCケモカインの産生増加を誘発し，直接血管内皮細胞にも結合してICAM-1の発現を上昇やKCケモカインを放出し，活性化した好中球がMPOおよび活性酸素を産生することで，組織にとどまって二次的な血管内皮細胞障害を起こすことが考えられはじめた．そして，MPO・$gp91^{phox}$ホモログを有するDUOXがPeroxidaseドメインを有し，間質性肺炎の進展にもかかわり自己免疫発症との関連でも注目されている．

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32 血尿

北野病院腎臓内科：武曾恵理

1. 鑑別診断のポイント

1) 尿潜血反応とは—本当に血尿か？

患者が「尿が赤くて血が出ている」と言って来られた場合，まず本当に“血”が混入しているのかを鑑別することが必要である．現在行われている試験紙法による潜血反応は赤血球から出たヘモグロビンの鉄によるペルオキシダーゼ反応に対する擬性触媒反応を利用している．ミオグロビン尿では，尿潜血反応は陽性で，血球がないことがある（擬陽性）．また，患者がアスコルビン酸（ビタミンC）を常用している場合などには，擬陰性となるので注意が必要である．

2) 尿沈渣の得られ方と見方のポイント

尿潜血反応陽性の場合，次に尿沈渣による血尿の有無とその形状の確認に入る．できるだけ新鮮な尿を遠沈して，沈渣を顕微鏡で観察する．赤血球の個数が倍率400倍で5個以上を異常血尿とする．その赤血球が正常でいびつな形をしていないもの（isomorphic）と，こぶ状，断片状，ねじれ状などの変形があるもの（dysmorphic）に分類する．後者は変形赤血球で，時として赤血球が連なった円柱を伴うことがある．

2. 診断へのアプローチ（チャート参照）

試験紙法で潜血が陽性で，沈渣で赤血球が4個以下の場合，ヘモグロビン尿やミオグロビン尿を考える．

5個以上で，正常赤血球が散在している場合，外科的（尿路性）血尿が考えられる．この場合，腎膀胱超音波検査を行い，異常があればCTや膀胱鏡などの泌尿器科的検査に進む．また，悪性腫瘍の鑑別の為に，尿細胞診を行う．

一方，尿沈渣で変形赤血球があり，赤血球円柱や蛋白尿の合併等がある場合，内科的血尿を考える．高血圧の有無，血液生化学検査で補体，IgA値の異常がある場合，腎生検を行い，腎糸球体病変の診断に進む．この場合も，外科的疾患の合併や，また腎の萎縮の有無を見，腎生検の可能性も検討するため，腎膀胱超音波診断を行う．

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