all animal peroxidases, the functional or biological advantage of covalently bound heme is not known (73). Recent studies indicate that the presence of a single covalent bond between the heme group and its protein is sufficient to protect the heme vinyl group from modification by hypohalous acid generated by peroxidases (77–79). In this way, peroxidases such as MPO can generate large amounts of HOCl and its by-products within the neutrophil phagosome without incurring autoinactivation by modification of the heme vinyl groups and subsequent inactivation.

In this study we describe the impact of two novel mutations on the biosynthesis and function of human MPO. The target residues, Gly-501 and Arg-499, are present on the proximal side of the heme pocket and are conserved in all four members of the animal peroxidase family. Although both missense mutations compromised proper proteolytic processing and intracellular targeting of mature MPO, the precursors for each incorporated heme, albeit inefficiently. Both biosynthetic radiolabeling of heme (Fig. 8) and spectroscopy (Fig. 9) demonstrated the presence of small amounts of heme in the proproteins of G501S and R499C. However, the Soret band was blue-shifted in each, demonstrating a spectrum more akin to that seen with LPO, TPO, or EPO rather than that characteristic of native MPO. Although the spectra of the mutants were consistent with the presence of heme covalently bound, likely via two rather than three bonds, neither had enzymatic activity. Given the known electrostatic interaction between Arg-499 and the ring D propionates of the heme in MPO, we predicted that the loss of this critical arginine would itself compromise heme binding and stability. Furthermore, the presence of cysteine at this site might allow a strong polar or covalent interaction between the sulfur atom in Cys-499 and the heme iron, which could reduce the oxidation potential and enzymatic activity of the mutant MPO. In the case of changes at codon 501, glycine may be the only amino acid at that site that allows optimal heme binding, consistent with our spectral data indicating incorporation of only small amounts of heme. For any serine that is incorporated in the heme pocket, our modeling of G501S suggested that its dysfunction reflects the increased distance between the heme iron and its distal histidine at 502, which is predicted to be twice that present in normal MPO, consistent with the loss of one coordination site for the heme iron, as suggested experimentally by the spectroscopy.

Taken together, these studies of biosynthesis and biochemical characterization of mutant MPO expressed in HEK cells demonstrated the structural and functional impact of two naturally occurring missense mutations underlying hereditary myeloperoxidase deficiency. Future applications of these analytical techniques to other mutations, both experimentally created and spontaneously occurring, should provide novel insights regarding the structure-function relationships unique to the animal peroxidase protein family.

Acknowledgment—We thank Dr. Ramaswamy Subramanian for advice on mutational analysis.

REFERENCES

1. Klebanoff, S. J. (2005) *J. Leukocyte Biol.* **77,** 598–625
2. Nauseef, W. M. (2004) *Histochem. Cell Biol.* **122,** 277–291
3. Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998) *Blood* **92,** 3007–3017
4. Hazen, S. L., Hsu, F. F., Mueller, D. M., Crowley, J. R., and Heinecke, J. W. (1996) *J. Clin. Investig.* **98,** 1283–1289
5. Hazen, S. L., Hsu, F. F., and Heinecke, J. W. (1996) *J. Biol. Chem.* **271,** 1861–1867
6. Henderson, J. P., Byun, J., and Heinecke, J. W. (1999) *J. Biol. Chem.* **274,** 33440–33448
7. Hazen, S. L., d'Avignon, A., Anderson, M. M., Hsu, F. F., and Heinecke, J. W. (1998) *J. Biol. Chem.* **273,** 4997–5005
8. Hazen, S. L., Hsu, F. F., d'Avignon, A., and Heinecke, J. W. (1998) *Biochemistry* **37,** 6864–6873
9. Domigan, N. M., Charlton, T. S., Duncan, M. W., Winterbourn, C. C., and Kettle, A. J. (1995) *J. Biol. Chem.* **270,** 16542–16548
10. Winterbourn, C. C., Pichorner, H., and Kettle, A. J. (1997) *Arch. Biochem. Biophys.* **338,** 15–21
11. Fiedler, T. J., Davey, C. A., and Fenna, R. E. (2000) *J. Biol. Chem.* **275,** 11964–11971
12. Furtmüller, P. G., Zederbauer, M., Jantschko, W., Helm, J., Bogner, M., Jakopitsch, C., and Obinger, C. (2006) *Arch. Biochem. Biophys.* **445,** 199–213
13. Furtmüller, P. G., Burner, U., and Obinger, C. (1998) *Biochemistry* **37,** 17923–17930
14. Marquez, L. A., and Dunford, H. B. (1994) *J. Biol. Chem.* **269,** 7950–7956
15. Furtmüller, P. G., Obinger, C., Hsuanyu, Y., and Dunford, H. B. (2000) *Eur. J. Biochem.* **267,** 5858–5864
16. Marchetti, C., Patriarca, P., Solero, G. P., Baralle, F. E., and Romano, M. (2004) *Hum. Mutat.* **23,** 496–505
17. DeLeo, F. R., Goedken, M., McCormick, S. J., and Nauseef, W. M. (1998) *J. Clin. Investig.* **101,** 2900–2909
18. Nauseef, W. M., Cogley, M., and McCormick, S. (1996) *J. Biol. Chem.* **271,** 9546–9549
19. Nauseef, W. M., McCormick, S., and Goedken, M. (2000) *Redox Rep.* **5,** 197–206
20. Hansson, M., Olsson, I., and Nauseef, W. M. (2006) *Arch. Biochem. Biophys.* **445,** 214–224
21. Ohashi, Y. Y., Kameoka, Y., Persad, A. S., Koi, F., Yamagoe, S., Hashimoto, K., and Suzuki, K. (2004) *Gene (Amst.)* **327,** 195–200
22. Persad, A. S., Kameoka, Y., Kanda, S., Niho, Y., and Suzuki, K. (2006) *Gene Expr.* **13,** 1–5
23. Nauseef, W. M., Root, R. K., and Malech, H. L. (1983) *J. Clin. Investig.* **71,** 1297–1307
24. Nauseef, W. M., McCormick, S. J., and Clark, R. A. (1995) *J. Biol. Chem.* **270,** 4741–4747
25. MacKerell, A. D., Bashford, D., Bellott, M., Dunbrack, R. L., Jr., Evanseck, J. D., Field, M. J., Fischer, S., Gao, J., Guo, H., Ha, S., Joseph-McCarthy, D., Kuchnir, L., Kuczera, K., Lau, F. T. K., Mattos, C., Michnick, S., Ngo, T., Nguyen, D. T., Prodhom, B., Reiher, W. E., III, Roux, B., Schlenkrich, M., Smith, J. C., Stote, R., Straub, J., Watanabe, M., Wiórkiewicz-Kuczera, J., Yin, D., and Karplus, M. (1998) *J. Phys. Chem. B* **102,** 3586–3616
26. Nauseef, W. M., McCormick, S. J., and Goedken, M. (1998) *J. Biol. Chem.* **273,** 7107–7111
27. Bülow, E., Nauseef, W. M., Goedken, M., McCormick, S., Calafat, J., Gullberg, U., and Olsson, I. (2002) *J. Leukocyte Biol.* **71,** 279–288
28. Nauseef, W. M., McCormick, S., and Yi, H. (1992) *Blood* **80,** 2622–2633
29. Nauseef, W. M., Olsson, I., and Strömberg-Arnljots, K. (1988) *Eur. J. Haematol.* **40,** 97–110
30. Nauseef, W. M. (1986) *Blood* **67,** 865–872
31. Nauseef, W. M. (1987) *Blood* **70,** 1143–1150
32. Nauseef, W. M., and Clark, R. A. (1986) *Blood* **68,** 442–449
33. Olsen, R. L., and Little, C. (1983) *Biochem. J.* **209,** 781–787
34. Pember, S. O., Shapira, R., and Kindade, J. M., Jr. (1983) *Arch. Biochem. Biophys.* **221,** 391–403
35. Van Dalen, C. J., Whitehouse, M. W., Winterbourn, C. C., and Kettle, A. J.

(1997) *Biochem. J.* **327,** 487–492
36. Kettle, A. J., and Winterbourn, C. C. (1994) *Methods Enzymol.* **233,** 502–512
37. Beers, R. J., and Sizer, I. W. (1952) *J. Biol. Chem.* **195,** 133–140
38. Dypbukt, J. M., Bishop, C., Brooks, W. M., Thong, B., Eriksson, H., and Kettle, A. J. (2005) *Free Radic. Biol. Med.* **39,** 1468–1477
39. Andersson, E., Hellman, L., Gullberg, U., and Olsson, I. (1998) *J. Biol. Chem.* **273,** 4747–4753
40. Borregaard, N., Heiple, J. M., Simons, E. R., and Clark, R. A. (1983) *J. Cell Biol.* **97,** 52–61
41. Bos, A. J., Wever, R., and Roos, D. (1978) *Biochim. Biophys. Acta* **525,** 37–44
42. Kameoka, Y., Persad, A. S., and Suzuki, K. (2004) *Jpn. J. Infect. Dis.* **57,** S12–S13
43. Battistuzzi, G., Bellei, M., Zederbauer, M., Furtmüller, P. G., Sola, M., and Obinger, C. (2006) *Biochemistry* **45,** 12750–12755
44. Helenius, A., Trombetta, E. S., Hebert, D. N., and Simons, J. F. (1997) *Trends Cell Biol.* **7,** 193–200
45. Arnljots, K., and Olsson, I. (1987) *J. Biol. Chem.* **262,** 10430–10434
46. Erdbrügger, U., Hellmark, T., Bunch, D. O., Alcorta, D. A., Jennette, J. C., Falk, R. J., and Nachman, P. H. (2006) *Kidney Int.* **69,** 1799–1805
47. Klebanoff, S. J. (1999) *Proc. Assoc. Am. Physicians* **111,** 383–389
48. Elzanowska, H., Wolcott, R. G., Hannum, D. M., and Hurst, J. K. (1995) *Free Radic. Biol. Med.* **18,** 437–449
49. Wolcott, R. G., Franks, B. S., Hannum, D. M., and Hurst, J. K. (1994) *J. Biol. Chem.* **269,** 9721–9728
50. Hurst, J. K., and Lymar, S. V. (1999) *Acc. Chem. Res.* **32,** 520–528
51. Dunford, H. B. (1999) *Heme Peroxidases,* 1st Ed., pp. 528, Wiley-VCH, New York
52. Kimura, S., and Ikeda-Saito, M. (1988) *Proteins* **3,** 113–120
53. Zeng, J., and Fenna, R. E. (1992) *J. Mol. Biol.* **226,** 185–207
54. Moguilevsky, N. (2000) in *The Peroxidase Multigene Family of Enzymes* (Nauseef, W. M., and Petrides, P. E., eds) pp. 38–44, Springer-Verlag, New York
55. Moguilevsky, N., Garcia-Quintana, L., Jacquet, A., Tournay, C., Fabry, L., Piérard, L., and Bollen, A. (1991) *Eur. J. Biochem.* **197,** 605–614
56. Kooter, I. M., Moguilevsky, N., Bollen, A., van der Veen, L. A., Otto, C., Dekker, H. L., and Wever, R. (1999) *J. Biol. Chem.* **274,** 26794–26802
57. Nauseef, W. M. (1999) *J. Lab. Clin. Med.* **134,** 215–221
58. Nauseef, W. M. (2003) in *Calreticulin* (Eggleton, P., and Michalak, M., eds) pp. 63–74, Landes Bioscience, Georgetown, TX
59. Johnson, K. R., Nauseef, W. M., Care, A., Wheelock, M. J., Shane, S., Hudson, S., Koeffler, H. P., Selsted, M., Miller, C., and Rovera, G. (1987) *Nucleic Acids Res.* **15,** 2013–2028
60. Nauseef, W. M. (1989) *Blood* **73,** 290–295
61. Nauseef, W. M., Brigham, S., and Cogley, M. (1994) *J. Biol. Chem.* **269,** 1212–1216
62. Nauseef, W. M. (1998) *J. Mol. Med.* **76,** 661–668
63. Nauseef, W. M. (1988) in *Hematology/Oncology Clinics of North America* (Curnutte, J. T., ed) pp. 135–158, W. B. Saunders Co., Philadelphia
64. Yamada, M., Hur, S.-J., and Toda, H. (1990) *Biochem. Biophys. Res. Commun.* **166,** 852–859
65. Yamada, M., Mori, M., and Sugimura, T. (1981) *Biochemistry* **20,** 766–771
66. Kettle, A. J., and Winterbourn, C. C. (1990) *Biochim. Biophys. Acta* **1052,** 379–385
67. Zgliczynski, J. M., Stelmaszynska, T., Domanski, J., and Ostrowski, W. (1971) *Biochim. Biophys. Acta* **235,** 419–424
68. Strauss, R. R., Paul, B. B., Jacobs, A. A., and Sbarra, A. J. (1971) *Infect. Immun.* **3,** 595–602
69. Henderson, L. M., and Chappell, J. B. (1996) *Biochim. Biophys. Acta* **1273,** 87–107
70. DeGioia, L., Ghibaudi, E. M., Laurenti, E., Salmona, M., and Ferrari, R. P. (1996) *J. Biol. Inorg. Chem.* **1,** 476–485
71. Ferrari, R. P. (2000) in *The Peroxidase Multigene Family of Enzymes: Biochemical Basis and Clinical Applications* (Petrides, P. E., and Nauseef, W. M., eds) pp. 114–121, Springer-Verlag, Berlin
72. DePillis, G. D., Ozaki, S., Kuo, J. M., Maltby, D. A., and Ortiz de Montellano, P. R. (1997) *J. Biol. Chem.* **272,** 8857–8860
73. Colas, C., and Ortiz de Montellano, P. R. (2003) *Chem. Rev.* **103,** 2305–2332
74. Fayadat, L., Niccoli-Sire, P., Lanet, J., and Franc, J.-L. (1999) *J. Biol. Chem.* **274,** 10533–10538
75. Kooter, I. M., Moguilevsky, N., Bollen, A., Sijtsema, N. M., Otto, C., and Wever, R. (1997) *J. Biol. Inorg. Chem.* **2,** 191–197
76. Brogioni, S., Feis, A., Marzocchi, M. P., Zederbauer, M., Furtmüller, P. G., Obinger, C., and Smulevich, G. (2006) *J. Raman Spectrosc.* **37,** 263–276
77. Huang, L., Wojciechowski, G., and Ortiz de Montellano, P. R. (2006) *J. Biol. Chem.* **281,** 18983–18988
78. Huang, L., Wojciechowski, G., and Ortiz de Montellano, P. R. (2005) *J. Am. Chem. Soc.* **127,** 5345–5353
79. Huang, L., and de Montellano, P. R. O. (2006) *Arch. Biochem. Biophys.* **446,** 77–83

Microbiol. Immunol., 51(5), 551 566, 2007

Editor-Communicated Paper

Trafficking of QD-Conjugated MPO-ANCA in Murine Systemic Vasculitis and Glomerulonephritis Model Mice

Akiyoshi Hoshino[1,2,3], Tomokazu Nagao[1], Toshiko Ito-Ihara[1,4,5,6], Akiko Ishida-Okawara[1], Kazuko Uno[5], Eri Muso[6], Noriko Nagi-Miura[7], Naohito Ohno[7], Kazuhiro Tokunaka[8], Shiro Naoe[9], Hiroshi Hashimoto[10], Masato Yasuhara[3], Kenji Yamamoto[2,3], and Kazuo Suzuki*[,1]

[1]Department of Bioactive Molecules, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, 162 8640 Japan, [2]International Clinical Research Center, Research Institute, International Medical Center of Japan, Tokyo, Japan, [3]Department of Pharmacokinetics and Pharmacodynamics, Hospital Pharmacy, Tokyo Medical and Dental University Graduate School, Bunkyo-ku, Tokyo, 113 8520 Japan, [4]Department of Nephrology and Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, 606 8507 Japan, [5]Louis Pasteur Center for Medical Research, Kyoto, 606 8225 Japan, [6]Kitano Hospital Medical Research Institute, Osaka, 530 8480 Japan, [7]Laboratory for Immunopharmacology of Microbial Products, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo, 192 0392 Japan, [8]Nippon Kayaku Co., Chiyoda-ku, Tokyo, 102 8172 Japan, [9]Department of Pathology, Toho University Ohashi Hospital, Meguro-ku, Tokyo, 153 8515 Japan, and [10]Department of Rheumatology and Internal Medicine, Juntendo University School of Medicine, Bunkyo-ku, Tokyo, 113 8421 Japan

Communicated by Dr. Hidechika Okada: Received February 8, 2007. Accepted February 27, 2007

***Abstract*: In systemic vasculitis, the serum level of myeloperoxidase (MPO)-specific anti-neutrophil cytoplasmic autoantibodies (MPO-ANCA) is significantly elevated with the progression of disease. We have established a model of murine systemic vasculitis by administration of MPO-ANCA and fungal mannoprotein to C57BL/6 mice. We examined the role of MPO and MPO-ANCA in the pathogenesis of glomerulonephritis and systemic vasculitis in this model using quantum dots (QDs). We demonstrated that QD-conjugated MPO-ANCA (ANCA-QD) visualized the translocation of MPO on the neutrophil membrane surface after stimulation with proinflammatory cytokines. We also observed that MPO translocation on neutrophils in both patients with rapid progressive glomerulonephritis and these model mice without any stimulation, suggesting that MPO translocation is certain to contribute to the development of glomerular lesion. In addition, blood flow on the kidney surface vessel was significantly decelerated in both SCG/Kj mice and this model, suggesting that ANCA induces the damage of blood vessel. These results indicate that MPO-ANCA and surface-translocated MPO on the activated neutrophils coordinately plays essential roles in the initial steps of the glomerulonephritis.**

***Key words*: Neutrophils, Autoimmunity, Fungal, Autoantibodies, Cytokines**

Neutrophils act as the initial innate immune response against invading microorganisms to produce reactive oxygen species by releasing their lysosomal enzymes, including peroxidases (47, 48). One of them, myeloperoxidase (MPO), is an essential molecule in the initiation and execution of the acute inflammatory response and subsequent resolution of fungal, bacterial, and viral infection (1, 2, 8, 26, 32). However, excessive release of MPO is responsible for severe injury to

**Address correspondence to* Dr. Kazuo Suzuki, Department of Bioactive Molecules, National Institute of Infectious Diseases, Toyama 1 23 1, Shinjuku-ku, Tokyo 162 8640, Japan. Fax: +81 3 5285 1111. E-mail: ksuzuki@nih.go.jp

Abbreviations: ANCA, anti-neutrophil cytoplasmic autoantibody; CAWS, *C. albicans* water-soluble mannoprotein and β-glucan complex; H&E, hematoxylin/eosin; MPO, myeloperoxidase(s); MPO-ANCA, MPO-specific anti-neutrophil cytoplasmic autoantibody; PR-3, proteinase-3; QD, quantum dot; rmMPO, recombinant murine MPO; RPGN, rapidly progressive glomerulonephritis; SCG, spontaneous crescentic glomerulonephritis; SCG/Kj, SCG-forming mouse/Kinjoh.

organs and blood vessels; the neutrophils infiltrated into the inflammatory vascular lesion are believed to contribute to the progression of vasculitis (23). In particular, the MPO-specific anti-neutrophil cytoplasmic autoantibody (MPO-ANCA) is significantly involved in the development of various kinds of vasculitis, ANCA-associated rapid progressive glomerulonephritis (RPGN), and microscopic polyangiitis (11, 15, 30, 34). We and other groups have demonstrated that MPO is a major antigen for MPO-ANCA production by using MPO-deficient mice (3, 19), and adoptive transfer of MPO-reactive splenocytes into Rag2-deficient mice resulted in crescentic glomerulonephritis with high MPO-ANCA titer (53). Furthermore, we have shown the contribution of activated neutrophils in renal lesions using spontaneous crescentic glomerulonephritis-forming mouse/Kinjoh (SCG/Kj) glomerulonephritis model mice (12, 20). In the early phase of glomerulonephritis, the spontaneous release of MPO from neutrophils was increased, indicating that activated neutrophils contribute to the development of active crescentic lesion in SCG/Kj mice. On the other hand, it was reported that a murine vasculitis model, which is induced by the administration of *Candida albicans*-derived glycoprotein, was accompanied by a high titer of MPO-ANCA in serum as well as that of SCG/Kj (20). We also reported that administration of *Candida albicans* water-soluble mannoprotein (CAWS) induces coronary arteritis similar to Kawasaki Disease (36, 38). *C. albicans* infection shows a lethality resembling anaphylactic fungal sepsis due to the release of some types of polysaccharide fraction into the blood (28) and the subsequent production of proinflammatory cytokines (21). Thus, it is assumed that activated neutrophils may contribute to the development of ANCA-related systemic vasculitis due to the association of MPO-ANCA with MPO antigen (49).

Proteinase-3 (PR3), which is another autoantigen in neutrophils, is known to be translocated on the surface of activated neutrophils. In contrast, the surface translocation of MPO in living neutrophils has not been investigated morphologically by fluorescent microscopy, whereas detection by flow cytometric detection has been reported (14); detection of surface-translocated MPO on living neutrophils was difficult due to its oxidative enzymic activity (6). Therefore, a fluorescent probe with far-bright emission has been required for a long time to observe the MPO on living neutrophils. Recently, fluorescent nanocrystal quantum dots (QDs) have been applied to molecular biology because of their greater and far longer fluorescence; they are now widely used in biotechnological and medical applications (7, 10, 51, 52).

In the present study, we conjugated QDs with MPO-ANCA, and revealed the trafficking of MPO-ANCA *in vivo*. Thus, we here established the mouse models of systemic vascular inflammation to clarify the initial activation step of chronic autoimmune inflammation and systemic vasculitis.

Materials and Methods

Mice. C57BL/6J mice were purchased from Japan Clea, Inc. (Tokyo). SCG/Kj were provided by Nippon Kayaku Co. (Tokyo) and maintained in our animal facility at the National Institute of Infections Diseases. Male mice over 12 weeks old were used as aged mice. All experiments were performed according to the Guidelines for Laboratory Animal Experiments in Research and with the approval of the local ethics committee at the National Institute of Infectious Diseases.

Reagents. FMLP (formyl-Met-Leu-Phe oligopeptides) was purchased from the Peptide Institute (Osaka, Japan). Recombinant human TNF-α and IL-1β were purchased from Chemicon (Temecula, Calif., U.S.A.). Mouse complement was purchased from Rockland Immunochemicals, Inc. (Gilbertsville, Pa., U.S.A.). The MPO-ANCA used in study was polyclonal anti-rmMPO antibody (anti-rmMPO Ab) prepared by immunization with recombinant murine MPO, which is produced by *Escherichia coli* transfected with cDNA of murine MPO, as described previously (22, 36), and the produced MPO-ANCA have an ability to cross-react to both murine and human MPO molecules. QDs (ZnS-coated CdSe nanocrystal; approximately 642 nm red fluorescence) were synthesized chemically (17) and conjugated with MPO-ANCA via their sulfhydryl group using SMCC (Pierce Biotechnology, Rockfold, Ill., U.S.A.) (16). The QD-conjugated Ab produced bound physically to QDs (ANCA-QD) and was detected by transmission electron microscopy with osmium tetroxide (OsO_4)-negative staining. The antibody captured on QD measured with the Bradford reagent (Bio-Rad Laboratories, Hercules, Calif., U.S.A.) with the standard curve of bovine serum albumin indicated that each ANCA-QD had approximately 8.5 Abs per QD nanocrystal (data not shown, see ref. 18). The function of ANCA-QD was checked by fluorescent Western blotting and histochemical analysis (39). CAWS (*Candida albicans*-derived water-soluble mannoprotein and β-glucan complex) was extracted from the culture supernatant of *C. albicans* (IFO 1385) which was prepared as previously described (29).

Neutrophil extraction, surface MPO stain and flow cytometric analysis. Murine neutrophils were collected from the peritoneal cavity of C57BL/6 and the aged (13 wks, male) SCG/Kj mice 3 hr after i.p. injection with

8% casein/PBS. Human neutrophils were collected from a patient with MPO-ANCA-associated RPGN and a healthy control, as described previously (9). In brief, human neutrophils were isolated from heparinized peripheral blood with Lymphoprep™ (Axis-Shield, Dundee, U.K.), 1.5% dextran (200 kDa), and retained erythrocytes were removed with lysis buffer [0.75% NH_4Cl in 20 mM Tris-HCl (pH 7.6)]. Collected neutrophils were preincubated at 37 C with Hanks' balanced salt solution (HBSS) for 10 min, and plated on a prewarmed 12-μm-thick coverslip (Matsunami Glass Industries, Osaka, Japan) attached with a silicone-made rubber well plate for 10 min. After stimulation with proinflammatory cytokines, neutrophils were immediately fixed with 1% paraformaldehyde/PBS and permeabilized by 0.1% Triton-X 100 if needed. Neutrophils were then blocked for 30 min with 1% bovine serum albumin (Sigma Cat#A6003; without serum MPO molecules) and then stained with ANCA-QD. Images were acquired with a CCD camera DP-70 (Olympus, Japan) under fluorescent microscopy IX-81 (Olympus) equipped with a LP (>610 nm) filter unit. For flow cytometry, collected neutrophils were stimulated for 10 min, stained with ANCA-QD and anti-human CD11b mAb (clone D12, PharMingen BD, San Diego, Calif., U.S.A.). Unfixed neutrophils were immediately analyzed by FACS Calibur (BD Biosciences).

ANCA-induced systemic vasculitis model and movie caption (intravital fluorescence videomicroscopy). C57BL/6J (male, 9 wks old) mice were injected i.v. with 1 mg of MPO-ANCA after pretreatment with i.p. injection of CAWS (4 mg/mouse) on day 0. On day 5, MPO-ANCA (1 mg/mouse) were injected again as secondary stimuli (see Fig. 1a). For imaging, 250 μg of ANCA-QD was added to the naïve MPO-ANCA in the case of second infection. Polyclonal mouse IgG was used as a negative control. After secondary stimulation, mice were sacrificed moribund on day 10. Bladder urine was collected to measure leaked protein and MPO-ANCA.

For intravital fluorescence videomicroscopy, mice were anesthetized with s.c. injection of ketaral solution (85 mg/kg) supplemented with xylazine (17 mg/kg). Mice were set on the videomicroscopy stage at 37 C with a heating pad, and renal surface microcirculation on the exposed right kidney was observed through a flank incision with RITC-dextran probes (5 mg/kg, i.v.; Sigma). The movie was captured by a video camera with image intensifier (Hamamatsu Photonics Corp., Hamamatsu, Japan) and recorded by a DVD video recorder (Hitachi, Ltd., Tokyo). The status of the superficial blood flow was determined in each microvessel by the following criteria: (1) Normal, capillaries with fast blood flow in which each erythrocyte could not be recognized; (2) occlusion, capillaries completely occluded by erythrocytes; and (3) abnormal, capillaries that show slow blood flow or repeat flow and stop.

Histology. For immunohistochemical stain, hematoxylin/eosin (H&E), and Periodic Acid Schiff (PAS) histology, collected kidney, liver, lung, and spleen were immediately washed, fixed with 10% formaldehyde neutralized solution for 4 hr. After the organs were incubated with 20% sucrose overnight, the cryosection was sliced to 4-μm thickness and affixed to a glass slide (Matsunami Glass Industries). Neutrophils were stained with FITC-conjugated anti-Gr1 mAb (PharMingen BD) or FITC or phycoerythrin (PE)-conjugated anti-CD11b mAb M1/70 (PharMingen). The disease score of the kidney lesion was evaluated by area glomerulus area, number of neutrophil infiltrating into the glomerulus, and cell proliferation in the glomerulus, based on Fischer's protected least significance difference (PLSD) test.

Multiple cytokine assay. Twelve microliters of undiluted mouse serum samples were used for parallel detection of 18 cytokines and chemokines in murine serum using the Bio-Plex® Cytokine Assay 20-Plex kit (Bio-Rad Laboratories) according to the manufacturer's protocol, and analyzed by the Bio-Plex Luminex 100 XYP instrument for analysis. Cytokine concentrations were calculated using Bio-Plex Manager 3.0 software with a five-parameter curve-fitting algorithm applied for standard curve calculations.

Statistical analysis. Data presented as mean ± 1 SD were compared using the two-tailed student's *t*-test and Fischer's PLSD test (Fig. 3) with KaleidaGraph 4.0 (Synergy Software, Reading, Pa., U.S.A.). *P* values <0.05 were regarded as significant.

Results

Establishment of a Murine Systemic Vasculitis Model Associated with MPO-ANCA

We have previously demonstrated that MPO-ANCA is enhanced in SCG/Kj mice showing spontaneous glomerulonephritis (37). Hence, MPO-ANCA has a key role in the development of systemic inflammation. To clarify the role of MPO-ANCA at the initial phase of autoimmune diseases, we need to create a novel systemic vasculitis model associated with MPO-ANCA. We have established a murine coronary arteritis model using CAWS fungal mannoprotein to C57BL/6 (36, 38), and this model also increased ANCA titer at a slow rate. To mimic higher ANCA-titer of systemic inflammation, we injected MPO-ANCA to the CAWS-pre-

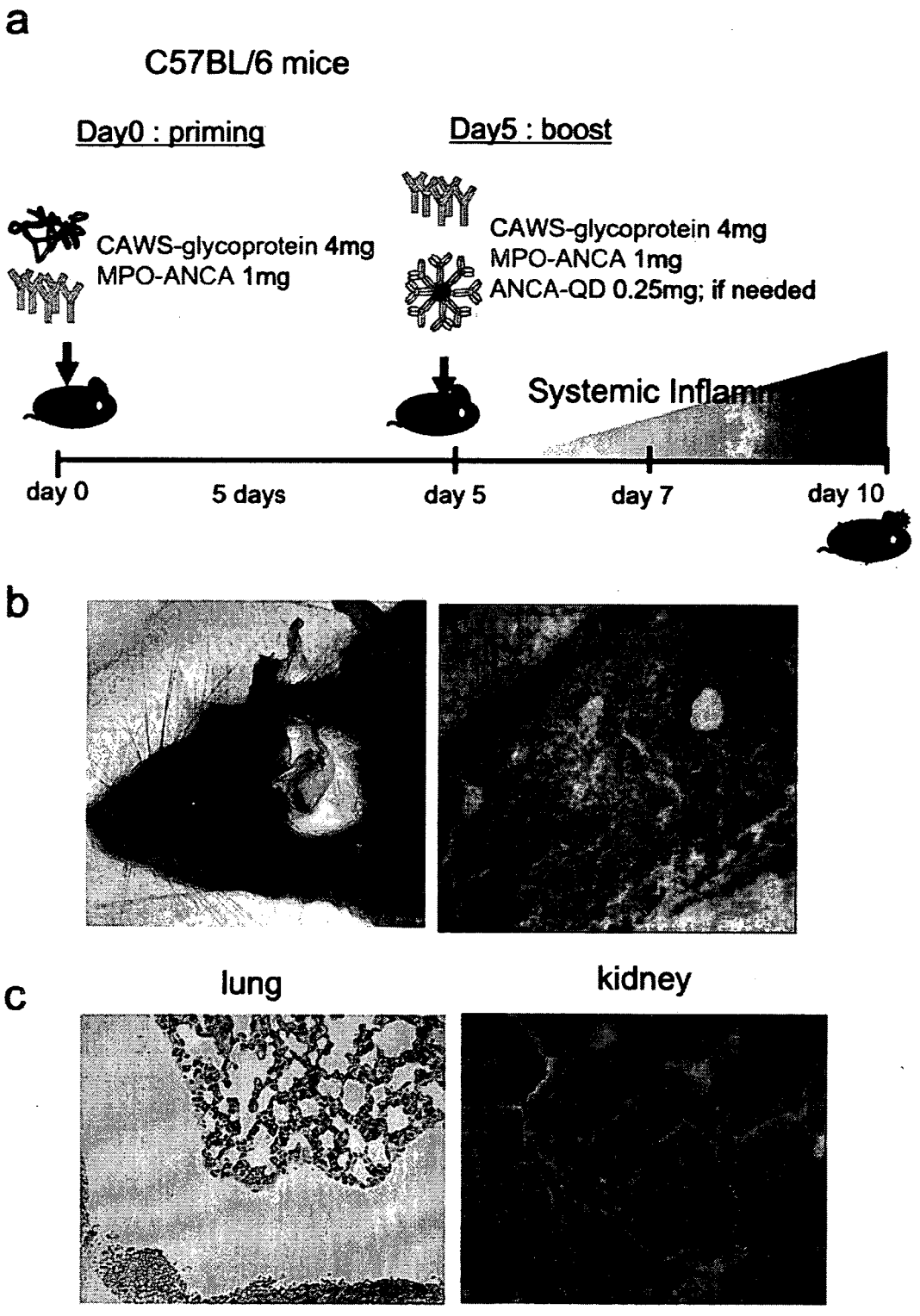

Fig. 1. Experimental procedure of MPO-ANCA-induced murine systemic vasculitis model. *a*, Schematic illustration of MPO-ANCA and CAWS-induced murine experimental systemic vasculitis model. C57BL/6J mice (male, 9 wks old) were injected i.v. with MPO-ANCA (1 mg of Ab/mouse) after pretreatment with 4 mg of i.p. injection of CAWS mannoprotein. On day 5, MPO-ANCA (1 mg naïve Ab/mouse, addition with an additional 250 μg/ml ANCA-QD if needed) was injected i.v. again to develop systemic vasculitis. *b*, Massive systemic inflammation in ear occurred in a mouse treated with CAWS and FMLP in addition to MPO-ANCA. The snapshots were taken 16 hr after secondary stimulation. Histochemical feature of inflamed auricle of systemic vasculitis mice was observed by PAS stain. Original magnification, ×200. *c*, H&E staining of the lung and kidney lesion of systemic vasculitis mice. Original magnification, ×200.

a

		0hr	3hr	18hr	24hr
Urinary protein [mg/dl]	Control IgG	<30	30	30	<30
	MPO-ANCA	<30	300	30-100	100
Occult blood	Control IgG	—	—	—	—
	MPO-ANCA	—	+	—	—

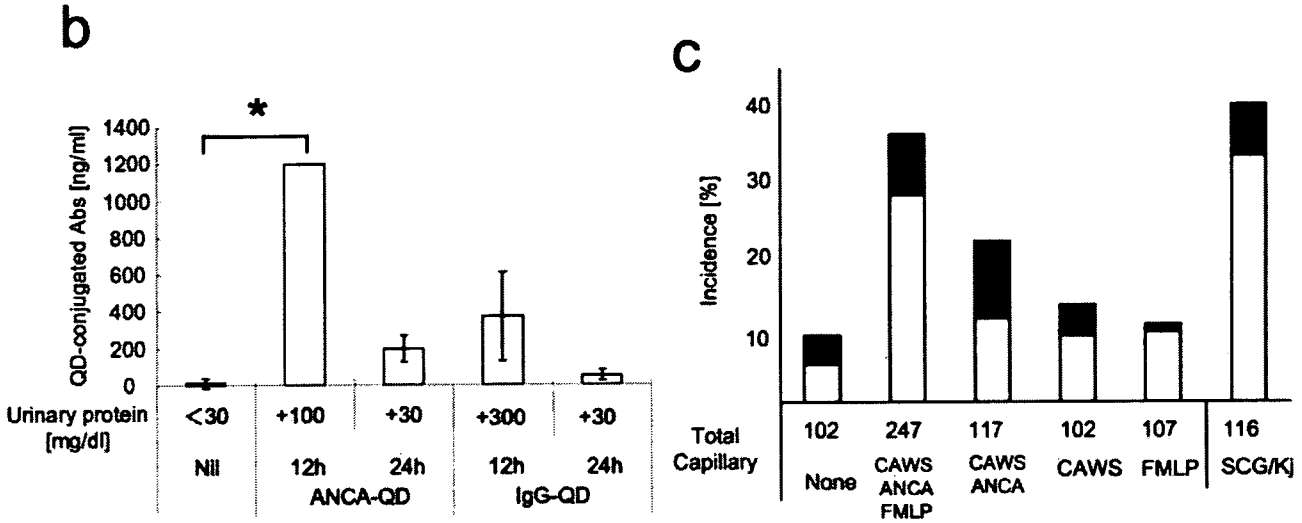

Fig. 2. Clinical feature of MPO-ANCA-induced systemic vasculitis mice. *a*, Urinary protein and occult blood leaked out from injured glomeruli were measured after second stimulation. *b*, Leaked ANCA-QD into urine was measured by fluorescent spectrometer. Ab concentration in collected urine was calculated based on the fluorescent standard curve of ANCA-QD. The data are presented as the mean ± standard deviation of duplicate samples (n=3). Total urinary protein was measured at the same time. *c*, The incidence of capillary with diminished blood flow (open column) and with the occlusion of renal circulation (filled column) was calculated. The counted capillary numbers in each kidney are displayed under each column.

treated mice in order to induce severe inflammation (Fig. 1a). As a result, massive systemic inflammation was observed, especially in severe necrotic auricularitis (Fig. 1b). In this model, approximately one-third mice died due to the hemorrhage inside the thorax (Fig. 1c). Moreover, histological analysis indicated that the inflammation was due to enhanced activation of infiltrating neutrophils in both lung and kidney (Fig. 1c). Next, we assessed the renal function of the glomerulonephritis. A renal dysfunction with massive proteinuria was detected from 2 hr after administration of MPO-ANCA (Fig. 2a). In addition, when ANCA-QD was additionally injected with MPO-ANCA in order to investigate the leaked autoantibody, QD-specific fluorescence was detected in the urine, whereas treatment with control IgG with CAWS promotes less proteinuria (Fig. 2b). To support this, in particular, the incidence ratio of capillary with diminished blood flow (open column) and with occlusion of kidney circulation (filled column) was increased after combined stimulation of MPO-ANCA and CAWS mannoprotein (Fig. 2c). The systemic inflammation was enhanced by costimulation of FMLP, implying that activated neutrophils significantly concerned with the glomerular lesion.

To further assess the systemic inflammation, histological staining of kidney and lung were performed. The neutrophils were infiltrated into glomeruli after MPO-ANCA injection (Fig. 3a, lower panel). With the infiltration of neutrophils, mesangiolysis with neutrophil infiltration was occurred in glomeruli on day 6, followed by significant spreading of the area of glomeruli (Fig. 3a and 3b). However, there is no intrinsic cell-proliferation in glomeruli (Fig. 3c), implying that MPO-ANCA injection with CAWS are associated with not mesangioproliferation but extensive widening of the subendothelial space, and mesangiolysis is thought to follow the endothelial injury (33).

Concurrently, massive pulmonary hemorrhage with

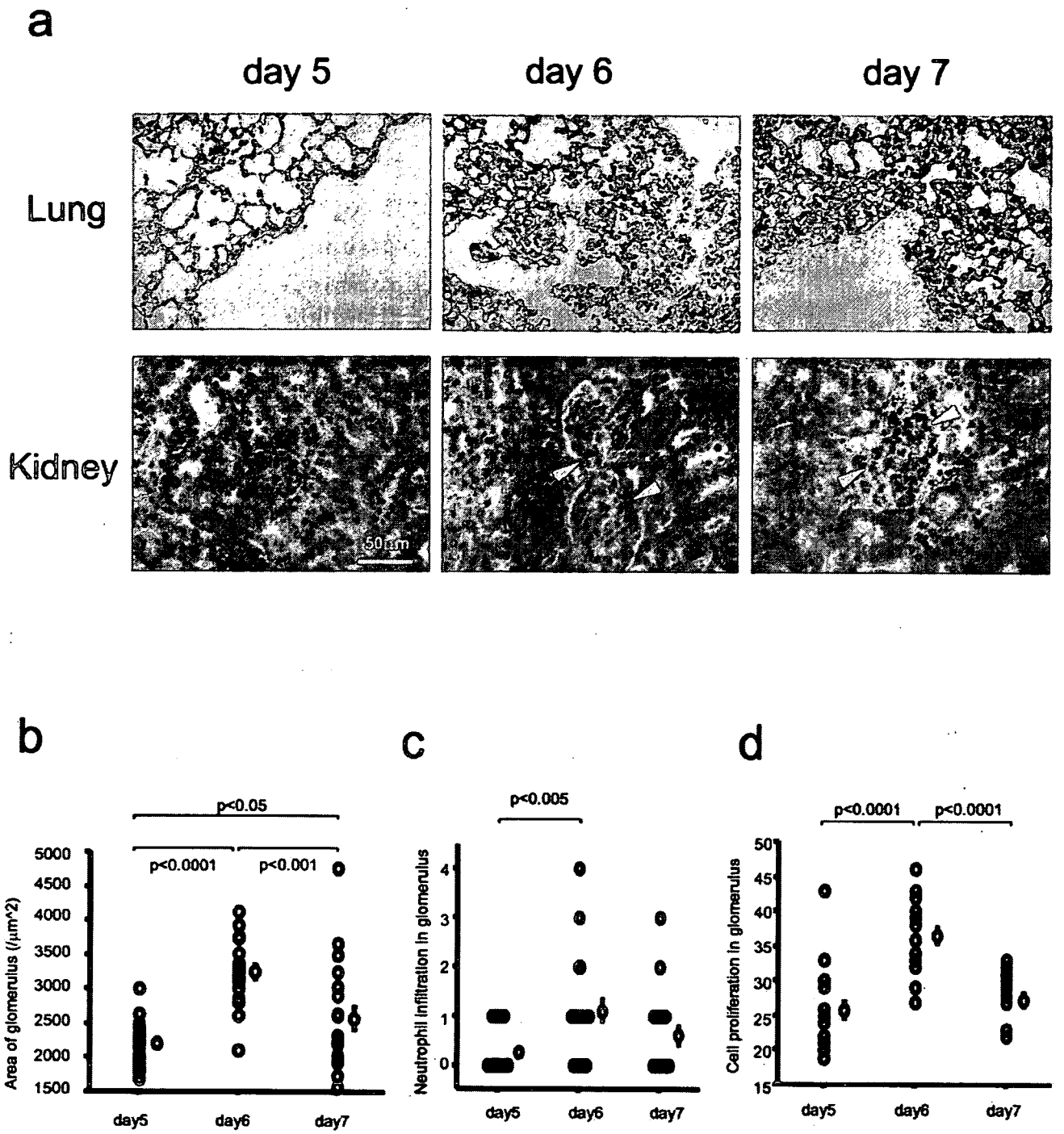

Fig. 3. Severe glomerular mesangiolysis with neutrophil infiltration was observed in experimental systemic vasculitis. PAS staining of kidney and lung in mice receiving MPO-ANCA (1 mg of naïve Ab with an additional 250 μg/ml of ANCA-QD for imaging). After the second injection with ANCA-QD, kidney and lung were collected on days 6 and 7, then embedded in paraffin sections to 4-μm thickness. (*a*) PAS staining of lung (upper) and kidney (lower). The arrowhead shows the infiltrated neutrophils. Magnification, ×400. (*b–d*) Histological score of glomerular lesions, neutrophil infiltration, and intrinsic cell proliferation in glomerulus in kidney were assessed. The data in red show an average ± standard deviation of kidney lesion (n=20). Significance in figures was calculated based on Fischer's PLSD test.

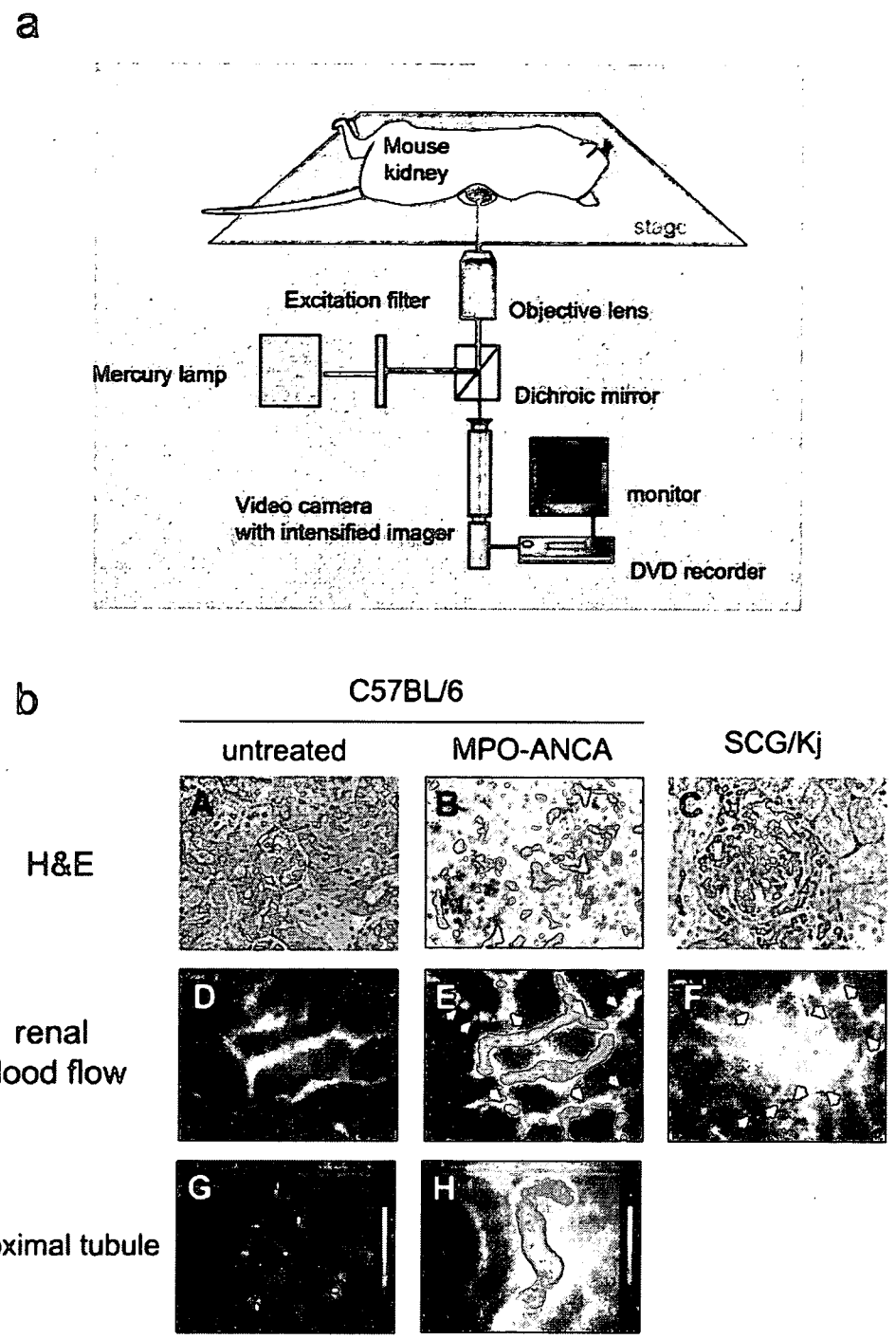

Fig. 4. MPO-ANCA administration with CAWS mannoprotein induces massive systemic vasculitis with severe renal blood deceleration. *a*, Schematic illustration of intravital videomicroscopy in MPO-ANCA-induced murine systemic vasculitis model. C57BL/6J mice were injected i.v. with MPO-ANCA twice (on day 0 and day 5) after pretreatment with 4 mg of i.p. CAWS injection. On day 10, intravital fluorescence videomicroscopy of renal surface microcirculation was performed on a fluorescent microscopy stage with a heating pad. Renal blood flow of systemic vasculitis mice described above was visualized by i.v. co-injection of RITC-dextran probes (5 mg/kg, i.v.; Sigma-Aldrich). *b*, H&E staining (top, *A–C*), renal blood flow (middle, *D–F*) of MPO-ANCA received and aged SCG/Kj mice developing glomerulonephritis. Arrowheads in H&E images indicate the infiltrated neutrophils. Original magnification, ×200. A representative histology from 10 examinations is presented and similar histological findings were obtained in other examinations. *Right*, time-lapse shots of renal blood flow by intravital fluorescent videomicroscopy. The arrows indicate leukocyte adhesion on the renal vessel. Serum proteins and MPO-ANCA were leaked out from collapsed glomeruli to the proximal tubule (bottom *G*, *H*). The RITC-dextran probe leaked to the proximal tubule in control mice and MPO-ANCA-received mice was detected.

severe neutrophil infiltration was observed in concert with the development of systemic inflammation (Fig. 3a, upper panel). In contrast, no inflammation was induced when control murine IgG were administered on day 5 (as a negative control IgG to CAWS-pretreated mice on day 0). In addition, treatment with only MPO-ANCA also showed neither lung inflammation nor mesangioproliferated glomeruli (data not shown). These result suggested that the combinational injection of MPO-ANCA with CAWS can induce the severe systemic inflammation with neutrophil infiltration.

Deceleration of Renal Blood Flow Due to Neutrophil Infiltration into Collapsed Glomeruli

To investigate whether vascular lesion with renal dysfunction occurred in kidney, we visualized the blood flow on the renal surface vessel using intravital fluorescent videomicroscopy (Fig. 4a). Simultaneous costimulation of MPO-ANCA dramatically decelerated blood flow in the kidney surface vessel with leukocyte adhesion, whereas mice administered only CAWS or MPO-ANCA alone still remained (Fig. 4b, inset E). Decelerated circulation was also observed in SCG/Kj mice without any stimulation (Fig. 4b, inset F). The dysfunction of kidney circulation also affected urogenous activity; a RITC-dextran probe injected into the circulating blood stream visualized the leakage of serum protein from the proximal tubule to the urine (Fig. 4b, inset G and H). This is consistent with results showing that a large amount of ANCA-QD was detected in urine (Fig. 2B). These results suggest that MPO-ANCA have a potential to cause glomerular lesion via neutrophil activation in concert with fungal mannoprotein.

Neutrophils of ANCA-Associated Glomerulonephritis Model Mice Shows Increased MPO Translocation to the Neutrophil Membrane

SCG/Kj mice are well known to spontaneously develop crescentic glomerulonephritis and systemic vasculitis (20, 25, 37), and we previously investigated in SCG/Kj mice the correlation of elevated ANCA titer after 10 weeks with the development of spontaneous vasculitis (36). Therefore, activated neutrophils were believed to translocate MPO on their surface as same as PR3. We stained neutrophils obtained from SCG/Kj mice, control C57BL/6, and ANCA-injected C57BL/6 mice with ANCA-QD. When neutrophils were stained with ANCA-QD, MPO was detected as translocated form on the surface membrane in almost 30% of neutrophils when they were primed with FMLP, whereas neutrophils of C57BL/6 mice without stimulation showed no MPO translocation (Fig. 5a). Thus, healthy murine neutrophils (C57BL/6) have an ability to translocate their concealed MPO to the plasma membrane only after priming with FMLP *in situ*. It is noteworthy that even nonstimulated neutrophils from both SCG/Kj and ANCA-treated C57BL/6 mice have translocated the MPO on their surface membrane. To support this, neutrophils of healthy volunteers and patients with ANCA-associated RPGN showed profiles similar to those of mice (data not shown). These observations indicate that the irregular MPO translocation on the neutrophils is involved in the MPO-ANCA-associated glomerulonephritis in mice as well as in humans. This result prompted us to investigate whether MPO translocated on the neutrophil surface by stimulation with CAWS and various proinflammatory cytokines, because ANCA-mediated neutrophil activation resulted in the generation of reactive oxygen species and degradation following cytokine production (42). Surface MPO translocation in normal human neutrophils was observed by FMLP and proinflammatory cytokines such as TNF-α, IL-1β and IL-8 (Fig. 5b). In addition, MPO translocation on neutrophils was observed at a concentration of 10^{-8} M FMLP, that is consistent with the result that human neutrophils show chemoattraction on FMLP at 10^{-8} M (18). However, CAWS stimulation induces no MPO translocation (Fig. 5b), implying that CAWS mannoprotein has no ability to activate neutrophils directly. These results indicate that the constitutive activation of neutrophils may be induced by proinflammatory cytokines on patients with ANCA-associated RPGN.

QD-Conjugated MPO-ANCA Are Detected on Activated Neutrophils in ANCA-Induced Systemic Vasculitis Mice

We visualized the trafficking of MPO-ANCA by the fluorescence of ANCA-QD. To investigate whether MPO-ANCA can be observed in inflammatory lesion, 1 mg of ANCA-QD were injected to the aged (13 wks old) SCG/Kj mice with developed glomerulonephritis. QD luminescence was widely distributed by blood flow and observed in organs including spleen, lung, liver, and kidney (Fig. 6a). Notably in kidney, ANCA-QD was specifically accumulated in the collapsed glomeruli 24 hr after administration of ANCA-QD into SCG/Kj mice (Fig. 6b). In contrast, when control IgG-QD were administered as a negative control antibody, no QD luminescence was observed in glomeruli despite the glomerulonephritis had already developed (Fig. 6c).

Next we visualized the trafficking of MPO-ANCA in our established murine systemic vasculitis model. To investigate whether MPO-ANCA is associated and reacted with activated neutrophils at glomerular lesion with neutrophil infiltration, 25% ANCA-QD were co-injected with MPO-ANCA when mice was stimulated

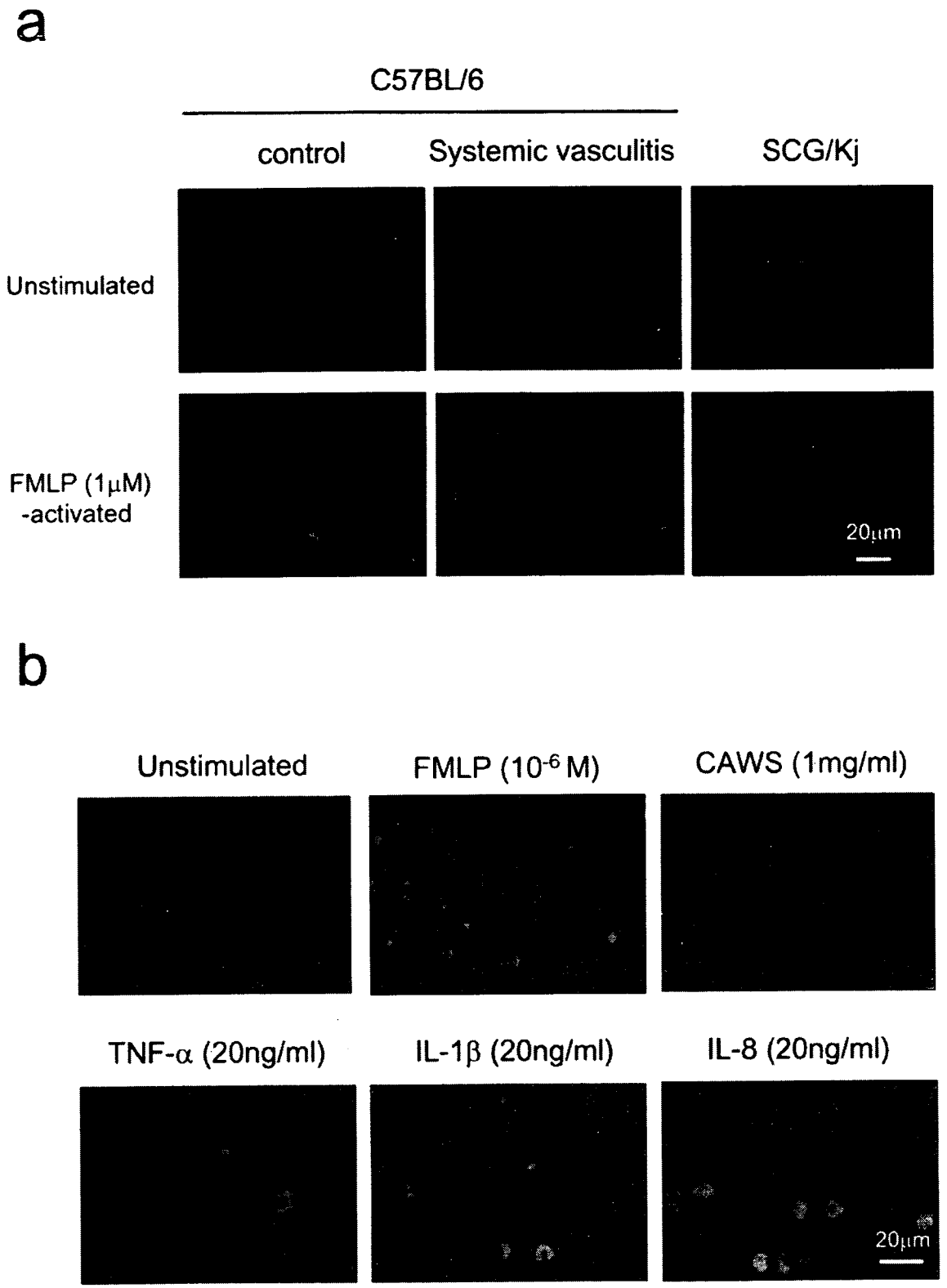

Fig. 5. MPO translocation on neutrophil membranes. The MPO translocation in murine and human neutrophils. *a*, Murine peritoneal neutrophils of naïve C57BL/6 mice, mice with ANCA-induced vasculitis, and aged (13 wks, male) SCG/Kj mice. Peripheral neutrophils were collected, stained with ANCA-QD with or without stimulation with FMLP. Bars indicate 20 μm. *b*, MPO surface translocation of human neutrophils after stimulation of proinflammatory cytokines. Human neutrophils (2×10^5 cells/ml) were stimulated with 1 μM FMLP, 1 mg/ml CAWS mannoprotein, 20 ng/ml TNF-α, 20 ng/ml IL-1β, and 20 ng/ml IL-8 at the indicated concentrations for 10 min, stained with ANCA-QD, and observed with fluorescent microscopy. Bar indicates 20 μm.

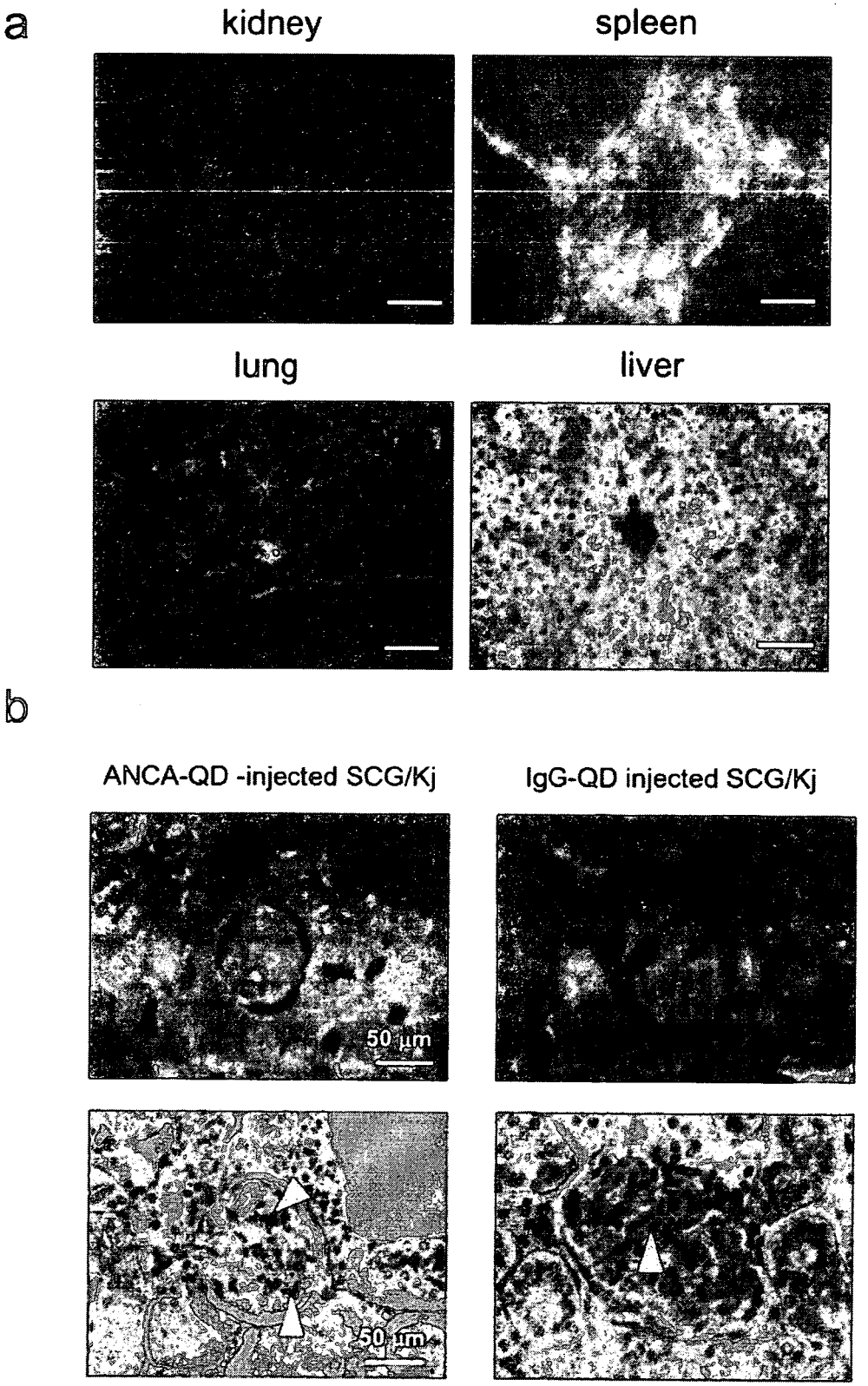

Fig. 6. Accumulation of ANCA-QD in the glomerulus with infiltrated neutrophils of SCG/Kj spontaneous glomerulonephritis mice. *a*, 1 mg of ANCA-QD were injected to the aged (13 wks old) SCG/Kj mice. The mice were sacrificed 24 hr after injection. Then collected spleen, lung, liver, and kidney were sliced to cryosection of 4 μm thickness. QD luminescence was observed with fluorescent microscopy. Bars indicate 250 μm. *b*, Fluorescent microscopic images (upper) and H&E stain (lower) of kidney cryosection of ANCA-QD-treated mice (left) and control IgG-QD-treated mice (right). Bars indicate 50 μm. The arrowheads indicate infiltrated segmented granulocytes.

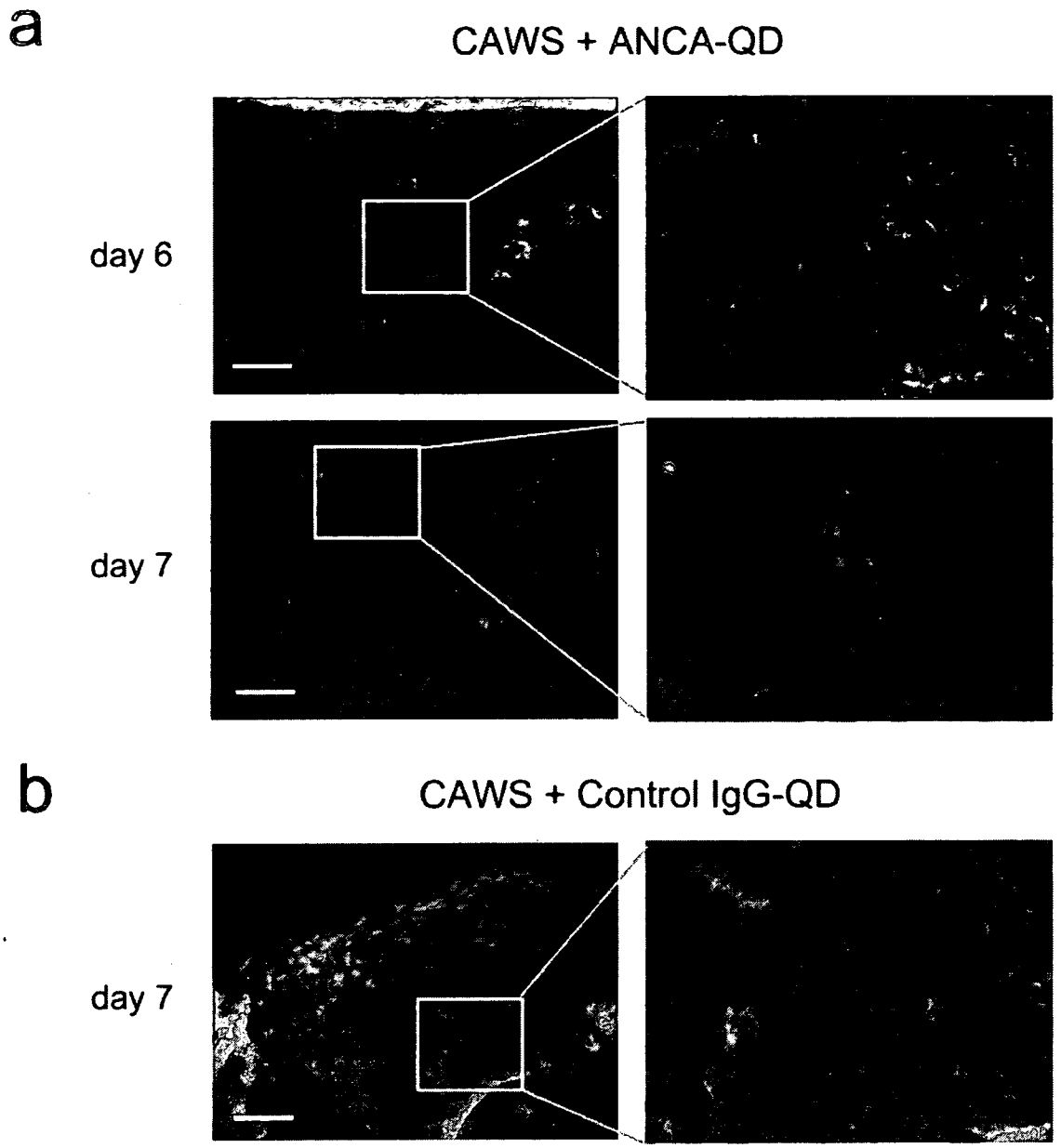

Fig. 7. Accumulation of ANCA-QD in the glomerulus with infiltrated neutrophils of MPO-ANCA-treated systemic vasculitis mice. Murine systemic vasculitis was induced in C57BL/6J mice as described in "Materials and Methods." In the case of second Ab injection, 250 μg of ANCA-QD were mixed with 1 mg of MPO-ANCA (*a*) or 250 μg of control IgG-QD were mixed with 1 mg of normal IgG, and injected to the primed mice. The mice were sacrificed 24 hr (day 6) and 48 hr (day 7) after second Ab-injection. Then collected kidney was sliced to cryosection of 4 μm thickness. Fluorescence emitted from ANCA-QD was observed with fluorescent microscopy. Fluorescent photos of right lane was enlarged images of gated area of left images. The bars indicate 250 μm.

secondary on day 5 (schematics is shown in Fig. 1a). After second stimulation with ANCA-QD, ANCA-QD was detected in developed glomerulonephritis lesion not on day 6 but on day 7 (Fig. 7a). In contrast, no signal was observed when control IgG-QD was injected (Fig. 7b). Treatment with only ANCA-QD without CAWS stimulation showed neither accumulation of ANCA-QD in kidney and lung nor mesangioproliferated glomeruli (data not shown).

To further investigate whether MPO-ANCA is reacted with activated neutrophils, immunohistochemical staining was performed. $CD11b^+$ $Gr1^+$ neutrophils were infiltrated in both lung (Fig. 8a) and glomeruli (Fig. 8b). However in kidney, ANCA-QD was not merged with infiltrated $Gr1^+$ cells but were located in the fringe of glomeruli. This result suggests that

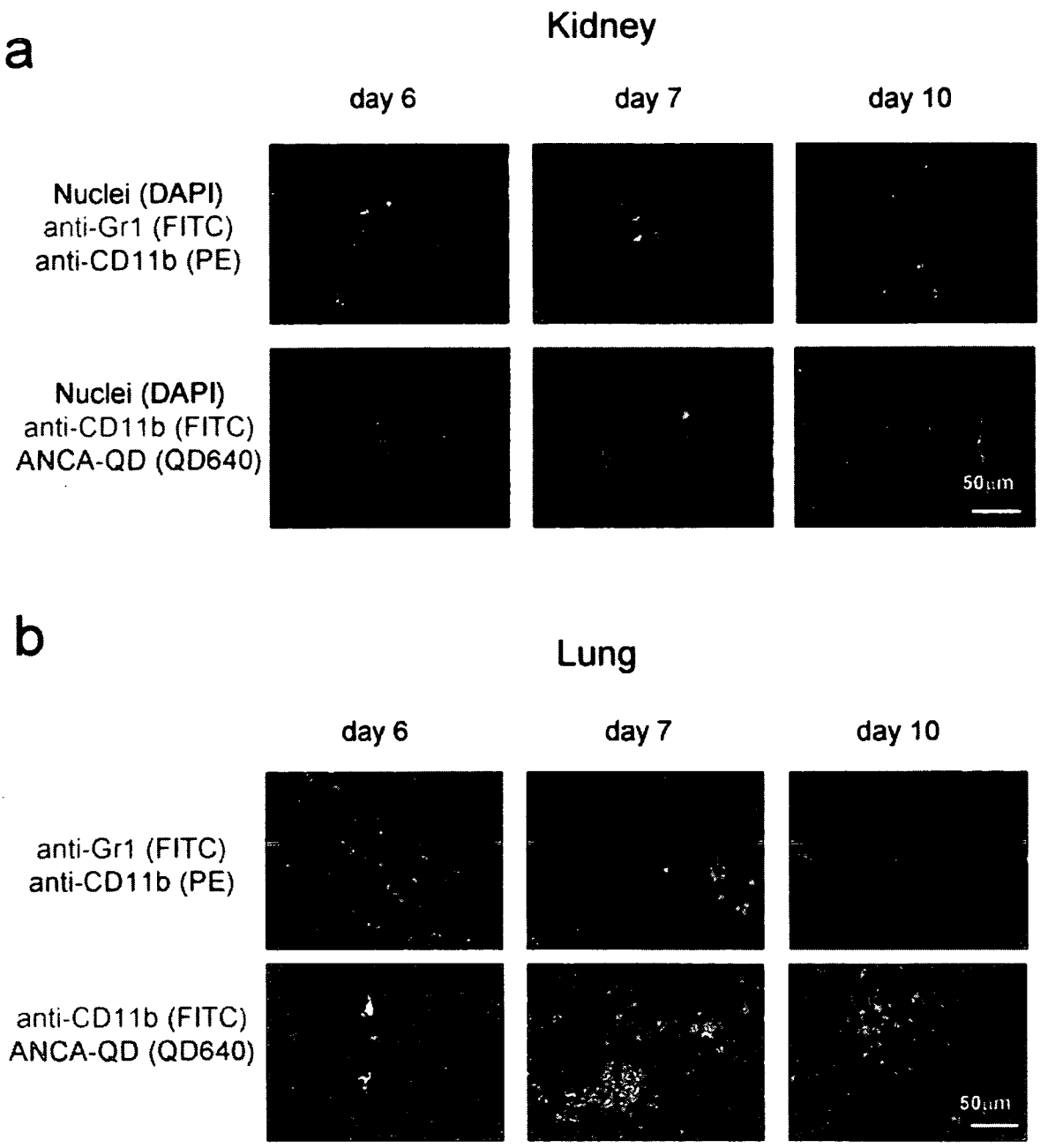

Fig. 8. ANCA-QD in renal glomerular lesion and pulmonary aggregation with infiltrated CD11b$^+$ and Gr1$^+$ neutrophils. Immunohistochemical stain of kidney and lung cryosections in mice receiving MPO-ANCA (1 mg of naïve Ab with an additional 250 μg/ml of ANCA-QD for imaging). *a*. Kidney lesion including injured glomeruli was collected on days 6, 7, and 10, then embedded in frozen sections and sliced to 4-μm thickness. The cryosection was stained with FITC anti-Gr1, PE anti-CD11b Ab (upper) and FITC anti-CD11b Ab (lower). ANCA-QD accumulated on the glomerular lesion was observed with fluorescent microscopy with a long-pass filter (>610-nm wavelength). The cryosection was stained with DAPI to display the location of the glomerulus. QD-positive cells were located around glomeruli. Original magnification, ×400. Bar indicates 50 μm. *b*. Injured lung collected on days 6, 7, and 10 was stained with FITC anti-Gr1, PE anti-CD11b Abs (upper), and FITC anti-CD11b Ab (lower), as described above. Large clusters of CD11b$^+$ and QD$^+$ are present in lung of day 10.

Table 1. Serum cytokine production of SCG/Kj mice

Cytokine conc. (pg/ml)	Control C57BL/6J	Anti-MPO treated C57BL/6J mice Day 5	Anti-MPO treated C57BL/6J mice Day 7	SCG/Kj
IL-1β	111.18 ± 13.44	141.01 ± 3.54	157.06 ± 7.78	207.41 ± 1.41
TNF-α	51.69 ± 9.65	169.46 ± 7.07	164.29 ± 8.49	1,064.07 ± 141.42
IL-6	12.56 ± 29.7	192.40 ± 21.92	547.33 ± 5.66	181.11 ± 63.64
IL-12(p40)[a]	4.77 ± 9.90	440.1 ± 19.8	302.35 ± 48.08	1,233.17 ± 115.26
IL-12(p70)[b]	21.52 ± 17.68	101.41 ± 33.94	90.54 ± 8.49	6.87 ± 0.71
IL-17	10.20 ± 7.78	39.81 ± 8.49	83.79 ± 5.66	106.58 ± 73.54
KC (CXCL1)	63.47 ± 18.31	783.7 ± 54.45	779.20 ± 88.39	414.7 ± 12.73
RANTES	264.59 ± 20.51	1,181.21 ± 84.85	940.52 ± 202.23	989.89 ± 137.18
G-CSF	42.77 ± 72.83	3,079.11 ± 113.14	461.43 ± 169.00	3,399.56 ± 111.72

The serum cytokine level of mice with MPO-ANCA-induced systemic vasculitis at the indicated time and that of SCG/Kj mice was measured by the Bio-Plex® cytokine detection kit. C57BL/6J mice were pretreated with CAWS and MPO-ANCA twice on days 0 and 5, as described in "Materials and Methods" (n=4). Serum cytokines of aged SCG/Kj mice developing glomerulonephritis (13 wks, male, n=4). The data are presented as the mean ± standard deviation of duplicate samples (n=4).

[a] IL-12p40 is detected by anti-IL-12p40 antibody.

[b] IL-12p70 is detected by the combination of anti-IL-12p35 and anti-IL-12p40 antibodies.

MPO-ANCA were not directly associated with the activated neutrophil. This is consistent with our previous results that MPO-ANCA was directly bound with glomerular endothelium and upregulated some adhesion molecules, including ICAM-1, VCAM-1, and E-selectin (35). In contrast, ANCA-QD was highly accumulated to the CD11b$^+$ macrophage cluster in lung inflammatory lesion (Fig. 8b). These results suggested that the infiltrated neutrophils in kidney and pulmonary lesion play a significant role in systemic vasculitis progression and inflammation. MPO-ANCA might act directly not to the neutrophils but to other cells, including mesangial and pulmonary epithelial cells.

Increased Cytokine Production in ANCA-Induced Systemic Vasculitis

Next, we investigated whether the serum cytokine level was increased and correlated with the neutrophil activations, because we have already revealed that neutrophils were activated and translocated MPO on membrane surface. Therefore, we exhaustively analyzed 18 kinds of cytokine production by Bioplex® analysis. Serum cytokine levels were dramatically changed with the development of vascular lesion. Proinflammatory cytokines, including IL-6, IL-1β and TNF-α and some neutrophil-related chemokines and growth factor including G-SCF, RANTES, and KC (murine functional IL-8), were significantly increased (Table 1). The anticipated secondary stimulants to activate neutrophils were due to those elevated cytokines such as G-CSF and RANTES that were released in response to the innate defense and subsequently drove some adhesion molecules, including integrin molecules and some chemokines, which was induced by belatedly produced cytokines such as IL-6. These results suggested that costimulation with MPO-ANCA and CAWS mannoprotein induce the neutrophil activation with MPO membrane translocation due to the produced proinflammatory cytokines.

Discussion

Several groups have examined that the histopathological features of systemic vasculitis caused in mice by stimulation with *C. albicans*-derived glycoprotein and the principal genetic roles in the development of coronary arterial vasculitis (36). It has also been reported that enzymatic activity of serum MPO level was elevated in neutrophils and marked higher ANCA titers in *Candida*-induced coronary vasculitis mice, similar to the same features in SCG/Kj mice (38). In addition, the ANCA titer is dramatically diminished in MPO-deficient mice (3, 19), indicating that fungal infection has relevance to the MPO molecule. Evidence suggests that MPO is an essential enzyme to protect fungal infection, and the overactivation of MPO could be a risk factor for arterial vasculitis formation. In the present study, we established a novel systemic vasculitis model by combination of mimicked higher ANCA titer by MPO-ANCA and mimicked fungal infection by CAWS. The fact that high levels of MPO-ANCA resulted in kidney dysfunction is consistent with the clinical feature that patients with ANCA-related RPGN showed significantly increased MPO activity and MPO-ANCA titer (14). These results suggest that the MPO-ANCA significantly participates in glomerulonephritis

in both mouse and human.

We have previously demonstrated that neutrophilic enzymatic activity is enhanced by stimulation with FMLP and proinflammatory cytokines. MPO was believed to exist not on the surface but inside neutrophils and seemed to leak MPO and other lysosomal peroxidase from the granules when neutrophils were activated (24). However, no direct evidence was observed to leak MPO on the neutrophil surface during neutrophil activation, whereas expression of PR3 on the surface of the primed neutrophils can be detected (4, 13, 50). The detection of MPO translocation on living neutrophils by QD is nothing short of groundbreaking. We assumed that the neutrophils in patients with ANCA-associated RPGN were constitutively activated by higher levels of proinflammatory cytokines and serum MPO. Our observation that neutrophils in patients translocate MPO on their surface without any stimulation supports this hypothesis. It is noteworthy that some healthy controls show lower MPO translocation on the surface of neutrophils even in unstimulated conditions in our experiments, and the ratio of MPO-translocated neutrophils also varied among patients and even in healthy controls. Similar features are also reported by PR3 expression levels on the surface of activated neutrophils; PR3 expression levels varied significantly in patients and even in healthy controls (44 46). These results indicate that resident translocation levels of MPO might be involved in the potential risk for developing MPO-ANCA-associated RPGN.

Visualization of MPO-ANCA in the early phase of systemic vasculitis by ANCA-QD provided a good deal of information about the initiation of neutrophil-mediated inflammation. Several researchers have noted that higher titers of ANCA mediate neutrophil responses in vascular inflammation directly and indirectly (5). Massive systemic inflammation, including nephritis and pulmonary hemorrhage, was observed with specific accumulation of ANCA-QD. Especially in kidney, immunohistochemical staining showed that neutrophils located around the fringe of glomeruli with the development of mesangiolysis. To support this, we have previously reported that MPO-ANCA directly bound to primary glomerular endothelium following upregulation of ICAM-1, VCAM-1, E-selectin, and promotion of TNF-α production (35). These results are consistent with the observation of Little et al. (31) that MPO-ANCA on leukocytes interacts with vascular endothelium via ICAM-1 in systemic vasculitis rats with developed MPO-ANCA. We also observed severe oliguria subsequently due to the mesangioproliferation with capillary obstruction on the glomeruli. The interaction among neutrophils, endothelium and MPO-ANCA promotes cytokine production from endothelial and/or mesangial cells around infiltrated neutrophil in the glomeruli, resulting in epithelial cell lysis, including mesangiolysis and interstitial hemorrhage, and consequently systemic vasculitis (40, 41, 43).

We hypothesized that the developmental time course of this systemic vasculitis seemed to be stepwise : 1) CAWS induced neutrophil priming following cytokine production with ANCA, 2) ANCA mediated neutrophil/endothelium activation. We demonstrated that peritoneal neutrophils have a potential to respond to MPO-ANCA and induce proinflammatory cytokines. MPO-ANCA and CAWS cooperatively promotes the production of proinflammatory cytokines, including IL-1, IL-6, IL-8 (in human), and TNF-α by a subset of macrophages and epithelial cells (27). However, we observed no MPO translocation on the neutrophil surface despite CAWS stimulation, implying that stimulation with only CAWS is insufficient to initiate neutrophil activation. Furthermore, CAWS have an ability to induce IL-6 by peritoneal neutrophils, subsequently resulting in enhanced neutrophil activation on day 7. In addition to CAWS, MPO-ANCA also might be contributed to neutrophil activation, because in our systemic vasculitis model we injected MPO-ANCA to mimic a higher ANCA titer just as in human RPGN patients, but CAWS alone failed to induce proinflammatory cytokine production (data not shown). In addition, only administration of MPO-ANCA promotes a lower level of IL-12p40 and G-CSF production (data not shown), implying a higher ANCA titer than in conventional conditions is a trigger to induce proinflammatory cytokine production in response to fungal infection. These results suggested that cytokine production in this model was promoted by at least two separate pathways: via MPO-ANCA specific activation and the CAWS-mediated pathway. A higher ANCA titer, established by repeated fungal infection, may be a potential risk for a patient who has a hereditary factor for autoimmune disease onset.

We conclude that the initial step of systemic vasculitis is due to the enhanced activation of neutrophils by CAWS and MPO-ANCA. CAWS and MPO-ANCA cooperatively trigger neutrophil activation by producing proinflammatory cytokines.

This work was supported primarily by grants from the Ministry of Health, Labour and Welfare, Japan and the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research, Japan (K. Suzuki), by a grant from the Japan Health Sciences Foundation on "Research on Health Sciences focusing on Drug Innovation, International Collaborative Research" (H. Hashimoto), partially

by a grant from Medical Techniques Promotion Research (H14-nano-004) from the Ministry of Health, Labour and Welfare, Japan (K. Yamamoto), and also partially by a grant for young researchers from the Japan Foundation of Cardiovascular Research (A. Hoshino). The author thanks Dr. Taeko Dohi (Department of Gastroenterology, Research Institute, International Medical Center of Japan), Dr. Yasuhiro Natori, and Ms. Mikiko Uwano (Department of Clinical Pharmacology, Research Institute, IMCJ) for kind and valuable advice and for help with the histochemical experiments on kidney.

References

1) Aratani, Y., Kura, F., Watanabe, H., Akagawa, H., Takano, Y., et al. 2002. Critical role of myeloperoxidase and nicotinamide adenine dinucleotide phosphate-oxidase in high-burden systemic infection of mice with *Candida albicans*. J. Infect. Dis. **185**: 1833 1837.
2) Aratani, Y., Kura, F., Watanabe, H., Akagawa, H., Takano, Y., et al. 2002. Relative contributions of myeloperoxidase and NADPH-oxidase to the early host defense against pulmonary infections with *Candida albicans* and *Aspergillus fumigatus*. Med. Mycol. **40**: 557 563.
3) Aratani, Y., Kura, F., Watanabe, H., Akagawa, H., Takano, Y., et al. 2000. Differential host susceptibility to pulmonary infections with bacteria and fungi in mice deficient in myeloperoxidase. J. Infect. Dis. **182**: 1276 1279.
4) Ben-Smith, A., Dove, S.K., Martin, A., Wakelam, M.J., and Savage, C.O. 2001. Antineutrophil cytoplasm autoantibodies from patients with systemic vasculitis activate neutrophils through distinct signaling cascades: comparison with conventional Fcgamma receptor ligation. Blood **98**: 1448 1455.
5) Csernok, E. 2003. Anti-neutrophil cytoplasmic antibodies and pathogenesis of small vessel vasculitides. Autoimmun. Rev. **2**: 158 164.
6) Csernok, E., Ernst, M., Schmitt, W., Bainton, D.F., and Gross, W.L. 1994. Activated neutrophils express proteinase 3 on their plasma membrane *in vitro* and *in vivo*. Clin. Exp. Immunol. **95**: 244 250.
7) Dubertret, B., Skourides, P., Norris, D.J., Noireaux, V., Brivanlou, A.H., and Libchaber, A. 2002. *In vivo* imaging of quantum dots encapsulated in phospholipid micelles. Science **298**: 1759 1762.
8) El Messaoudi, K., Verheyden, A.M., Thiry, L., Fourez, S., Tasiaux, N., et al. 2002. Human recombinant myeloperoxidase antiviral activity on cytomegalovirus. J. Med. Virol. **66**: 218 223.
9) Fujii, A., Tomizawa, K., Arimura, Y., Nagasawa, T., Ohashi, Y.Y., et al. 2000. Epitope analysis of myeloperoxidase (MPO) specific anti-neutrophil cytoplasmic autoantibodies (ANCA) in MPO-ANCA-associated glomerulonephritis. Clin. Nephrol. **53**: 242 252.
10) Gao, X., Cui, Y., Levenson, R.M., Chung, L.W., and Nie, S. 2004. *In vivo* cancer targeting and imaging with semiconductor quantum dots. Nat. Biotechnol. **22**: 969 976.
11) Gross, W.L., Schmitt, W.H., and Csernok, E. 1993. ANCA and associated diseases: immunodiagnostic and pathogenetic aspects. Clin. Exp. Immunol. **91**: 1 12.
12) Hamano, Y., Tsukamoto, K., Abe, M., Sun, G.D., Zhang, D., et al. 2006. Genetic dissection of vasculitis, myeloperoxidase-specific antineutrophil cytoplasmic autoantibody production, and related traits in spontaneous crescentic glomerulonephritis-forming/Kinjoh mice. J. Immunol. **176**: 3662 3673.
13) Harper, L., Cockwell, P., Adu, D., and Savage, C.O. **2001**. Neutrophil priming and apoptosis in anti-neutrophil cytoplasmic autoantibody-associated vasculitis. Kidney Int. **59**: 1729 1738.
14) Harper, L., Radford, D., Plant, T., Drayson, M., Adu, D., and Savage, C.O. 2001. IgG from myeloperoxidase-anti-neutrophil cytoplasmic antibody-positive patients stimulates greater activation of primed neutrophils than IgG from proteinase 3-antineutrophil cytosplasmic antibody-positive patients. Arthritis Rheum. **44**: 921 930.
15) Heeringa, P., Huugen, D., and Tervaert, J.W. 2005. Anti-neutrophil cytoplasmic autoantibodies and leukocyte-endothelial interactions: a sticky connection? Trends Immunol. **26**: 561 564.
16) Hoshino, A., Fujioka, K., Oku, T., Nakamura, S., Suga, M., et al. 2004. Quantum dots targeted to the assigned organelle in living cells. Microbiol. Immunol. **48**: 985 994.
17) Hoshino, A., Fujioka, K., Oku, T., Suga, M., Sasaki, Y.F., et al. 2004. Physicochemical properties and cellular toxicity of nanocrystal quantum dots depend on their surface modification. Nano Lett. **4**: 2163 2169.
18) Hoshino, A., Nagao, T., Nakasuga, A., Ishida-Okawara, A., Suzuki, K., et al. 2007. Nanocrystal quantum dot-conjugated anti-myeloperoxidase antibody as the detector of activated neutrophils. IEEE Transactions on NanoBioscience, in press.
19) Ichimori, K., Fukuyama, N., Nakazawa, H., Aratani, Y., Koyama, H., et al. 2003. Myeloperoxidase has directly-opposed effects on nitration reaction—study on myeloperoxidase-deficient patient and myeloperoxidase-knockout mice. Free Radic. Res. **37**: 481 489.
20) Ishida-Okawara, A., Ito-Ihara, T., Muso, E., Ono, T., Saiga, K., et al. 2004. Neutrophil contribution to the crescentic glomerulonephritis in SCG/Kj mice. Nephrol. Dial. Transplant. **19**: 1708 1715.
21) Ishida-Okawara, A., Nagi-Miura, N., Oharaseki, T., Takahashi, K., Okumra, A., et al. 2007. Neutrophil activation and arteritis induced by *C. albicans* water-soluble mannoprotein-beta-glucan complex (CAWS). Exp. Mol. Pathol. In press.
22) Ishida-Okawara, A., Oharaseki, T., Takahashi, K., Hashimoto, Y., Aratani, Y., et al. 2001. Contribution of myeloperoxidase to coronary artery vasculitis associated with MPO-ANCA production. Inflammation **25**: 381 387.
23) Jennette, J.C., Falk, R.J., Andrassy, K., Bacon, P.A., Churg, J., et al. 1994. Nomenclature of systemic vasculitides. Proposal of an international consensus conference. Arthritis Rheum. **37**: 187 192.
24) Kawai, Y., Okawara, A.I., Okuyama, H., Kura, F., and Suzuki, K. 2000. Modulation of chemotaxis, O(2)(−) production and myeloperoxidase release from human polymorphonuclear leukocytes by the ornithine-containing lipid and the serineglycine-containing lipid of *Flavobacterium.*

FEMS Immunol. Med. Microbiol. **28**: 205 209.
25) Kinjoh, K., Kyogoku, M., and Good, R.A. 1993. Genetic selection for crescent formation yields mouse strain with rapidly progressive glomerulonephritis and small vessel vasculitis. Proc. Natl. Acad. Sci. U.S.A. **90**: 3413 3417.
26) Klebanoff, S.J., and Coombs, R.W. 1992. Viricidal effect of polymorphonuclear leukocytes on human immunodeficiency virus-1. Role of the myeloperoxidase system. J. Clin. Invest. **89**: 2014 2017.
27) Kolls, J.K., and Linden, A. 2004. Interleukin-17 family members and inflammation. Immunity **21**: 467 476.
28) Kurihara, K., Miura, N.N., Uchiyama, M., Ohno, N., Adachi, Y., et al. 2000. Measurement of blood clearance time by Limulus G test of Candida-water soluble polysaccharide fraction, CAWS, in mice. FEMS Immunol. Med. Microbiol. **29**: 69 76.
29) Kurihara, K., Shingo, Y., Miura, N.N., Horie, S., Usui, Y., et al. 2003. Effect of CAWS, a mannoprotein-beta-glucan complex of *Candida albicans*, on leukocyte, endothelial cell, and platelet functions *in vitro*. Biol. Pharm. Bull. **26**: 233 240.
30) Lesavre, P., Noel, L.H., Gayno, S., Nusbaum, P., Reumaux, D., et al. 1993. Atypical autoantigen targets of perinuclear antineutrophil cytoplasm antibodies (P-ANCA): specificity and clinical associations. J. Autoimmun. **6**: 185 195.
31) Little, M.A., Smyth, C.L., Yadav, R., Ambrose, L., Cook, H.T., et al. 2005. Antineutrophil cytoplasm antibodies directed against myeloperoxidase augment leukocyte-microvascular interactions *in vivo*. Blood **106**: 2050 2058.
32) Moguilevsky, N., Steens, M., Thiriart, C., Prieels, J.P., Thiry, L., and Bollen, A. 1992. Lethal oxidative damage to human immunodeficiency virus by human recombinant myeloperoxidase. FEBS Lett. **302**: 209 212.
33) Morita, T., Yamamoto, T., and Churg, J. 1998. Mesangiolysis: an update. Am. J. Kidney Dis. **31**: 559 573.
34) Muso, E., Ito-Ihara, T., Ono, T., Imai, E., Yamagata, K., et al. 2004. Intravenous immunoglobulin (IVIg) therapy in MPO-ANCA related polyangiitis with rapidly progressive glomerulonephritis in Japan. Jpn. J. Infect. Dis. **57**: S17 S18.
35) Nagao, T., Matsumura, M., Mabuchi, A., Ishida-Okawara, A., Koshio, O., et al. 2007. Up-regulation of adhesion molecule expression in glomerular endothelial cells by anti-myeloperoxidase antibody. Nephrol. Dial. Transplant. **22**: 77 87.
36) Nagi-Miura, N., Shingo, Y., Adachi, Y., Ishida-Okawara, A., Oharaseki, T., et al. 2004. Induction of coronary arteritis with administration of CAWS (*Candida albicans* water-soluble fraction) depending on mouse strains. Immunopharmacol. Immunotoxicol. **26**: 527 543.
37) Neumann, I., Birck, R., Newman, M., Schnulle, P., Kriz, W., et al. 2003. SCG/Kinjoh mice: a model of ANCA-associated crescentic glomerulonephritis with immune deposits. Kidney Int. **64**: 140 148.
38) Ohno, N. 2004. Murine model of Kawasaki disease induced by mannoprotein-beta-glucan complex, CAWS, obtained from *Candida albicans*. Jpn. J. Infect. Dis. **57**: S9 S10.
39) Ornberg, R.L., Harper, T.F., and Liu, H. 2005. Western blot analysis with quantum dot fluorescence technology: a sensitive and quantitative method for multiplexed proteomics. Nat. Methods **2**: 79 81.
40) Radford, D.J., Lord, J.M., and Savage, C.O. 1999. The activation of the neutrophil respiratory burst by anti-neutrophil cytoplasm autoantibody (ANCA) from patients with systemic vasculitis requires tyrosine kinases and protein kinase C activation. Clin. Exp. Immunol. **118**: 171 179.
41) Radford, D.J., Savage, C.O., and Nash, G.B. 2000. Treatment of rolling neutrophils with antineutrophil cytoplasmic antibodies causes conversion to firm integrin-mediated adhesion. Arthritis Rheum. **43**: 1337 1345.
42) Rarok, A.A., Limburg, P.C., and Kallenberg, C.G. 2003. Neutrophil-activating potential of antineutrophil cytoplasm autoantibodies. J. Leukoc. Biol. **74**: 3 15.
43) Savage, C.O., Pottinger, B.E., Gaskin, G., Pusey, C.D., and Pearson, J.D. 1992. Autoantibodies developing to myeloperoxidase and proteinase 3 in systemic vasculitis stimulate neutrophil cytotoxicity toward cultured endothelial cells. Am. J. Pathol. **141**: 335 342.
44) Schreiber, A., Busjahn, A., Luft, F.C., and Kettritz, R. 2003. Membrane expression of proteinase 3 is genetically determined. J. Am. Soc. Nephrol. **14**: 68 75.
45) Schreiber, A., Luft, F.C., and Kettritz, R. 2004. Membrane proteinase 3 expression and ANCA-induced neutrophil activation. Kidney Int. **65**: 2172 2183.
46) Schreiber, A., Otto, B., Ju, X., Zenke, M., Goebel, U., et al. 2005. Membrane proteinase 3 expression in patients with Wegener's granulomatosis and in human hematopoietic stem cell-derived neutrophils. J. Am. Soc. Nephrol. **16**: 2216 2224.
47) Suzuki, K. 2003. Neutrophil functions of patients with MPO-ANCA-related vasculitis. Intern. Med. **42**: 552 553.
48) Suzuki, K., Muso, E., and Nauseef, W.M. 2004. Contribution of peroxidases in host-defense, diseases and cellular functions. Jpn. J. Infect. Dis. **57**: S1 S2.
49) Suzuki, K., and Okazaki, T. 2004. Contribution of myeloperoxidase in vasculitis development. Jpn. J. Infect. Dis. **57**: S2 S3.
50) van der Geld, Y.M., Oost-Kort, W., Limburg, P.C., Specks, U., and Kallenberg, C.G. 2000. Recombinant proteinase 3 produced in different expression systems: recognition by anti-PR3 antibodies. J. Immunol. Methods **244**: 117 131.
51) Voura, E.B., Jaiswal, J.K., Mattoussi, H., and Simon, S.M. 2004. Tracking metastatic tumor cell extravasation with quantum dot nanocrystals and fluorescence emission-scanning microscopy. Nat. Med. **10**: 993 998.
52) Wu, X., Liu, H., Liu, J., Haley, K.N., Treadway, J.A., et al. 2003. Immunofluorescent labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots. Nat. Biotechnol. **21**: 41 46.
53) Xiao, H., Heeringa, P., Hu, P., Liu, Z., Zhao, M., et al. 2002. Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. J. Clin. Invest. **110**: 955 963.

Nephrol Dial Transplant (2007) 22: 77–87
doi:10.1093/ndt/gfl555
Advance Access publication 27 September 2006

Original Article

NDT
Nephrology Dialysis Transplantation

Up-regulation of adhesion molecule expression in glomerular endothelial cells by anti-myeloperoxidase antibody

Tomokazu Nagao[1], Mimiko Matsumura[1,2], Ayako Mabuchi[3], Akiko Ishida-Okawara[1], Osamu Koshio[4], Toshinori Nakayama[5], Haruyuki Minamitani[2] and Kazuo Suzuki[1]

[1]Department of Bioactive Molecules, National Institute of Infectious Diseases, Tokyo, Japan, [2]Graduate School of Science and Technology, Keio University, Yokohama, Japan, [3]Department of Physiology, University of Otago, Dunedin, New Zealand, [4]Department of Medicine, Teikyo University, Tokyo, Japan and [5]Graduate School of Medicine, Chiba University, Chiba, Japan.

Abstract

Background. Anti-neutrophil cytoplasmic antibody directed against myeloperoxidase (MPO-ANCA) has been implicated in pauci-immune crescentic glomerulonephritis. It stimulates primed neutrophils to adhere to glomerular endothelial cells (GECs), thereby releasing reactive oxygen and other toxic substances and ultimately damaging the GECs. Though, a pathogenic role for MPO-ANCA is not fully understood, we hypothesized that MPO-ANCA modulates GEC functions by the increases in expression of adhesion molecules.

Methods. A polyclonal rabbit anti-recombinant mouse MPO antibody (anti-rmMPO IgG) was evaluated in mouse GEC (mGEC) for its effect on adhesion molecule expression. The primary culture of mGEC was incubated with anti-rmMPO IgG or isotype control and the expression of intercellular adhesion molecules-1 (ICAM-1) was evaluated by real-time reverse transcription–polymerase chain reaction (RT–PCR) analysis and ICAM-1 cell ELISA.

Results. The real-time RT–PCR analysis showed that a treatment with 100 μg/ml anti-rmMPO IgG increased the expression of mRNAs for ICAM-1, vascular cell adhesion molecule-1 and E-selectin by approximately 12.5, 7.5 and 10.5-fold, respectively. ICAM-1 cell ELISA also substantiated increased expression of ICAM-1. This enhancement of ICAM-1 expression was mediated by the antigen specificity of anti-rmMPO IgG. In addition, there were several proteins in mGEC specifically immunoprecipitated with anti-rmMPO IgG.

Conclusions. These results showed that anti-MPO antibody activates not only neutrophils, but also GEC, indicating that anti-rmMPO IgG-induced direct activation of GEC contributes to neutrophil adhesion to GEC, thereby increasing glomerular neutrophil infiltration in initiation and progression of pauci-immune glomerulonephritis.

Keywords: adhesion molecules; crescentic glomerulonephritis; glomerular endothelial cells; MPO-ANCA

Introduction

Anti-neutrophil cytoplasmic antibody directed against myeloperoxidase (MPO-ANCA) are involved in the development of small vessel vasculitis such as idiopathic crescentic glomerulonephritis and microscopic polyangiitis [1–3]. MPO-ANCA has been used as a specific marker for these vasculitides, as evident from clinical observations that MPO-ANCA titres generally correlate with disease activity [4–6], although pathogenicity of the autoantibodies has not been clearly understood. Neutrophils have been reported to be a primary target of MPO-ANCA due to localization of MPO in the azurophilic granules of neutrophils and their translocation to plasma membrane upon priming with cytokines such as tumour necrosis factor-α (TNF-α) [7,8]. Therefore, previous studies have mainly focused on the signalling pathway in respiratory burst and degranulation of neutrophils stimulated with MPO-ANCA and initiation mechanisms of small vessel vasculitis by MPO-ANCA through neutrophil activation [9–12].

Adhesion of neutrophils to endothelial cells is an important step for neutrophil infiltration into glomeruli which is a histological feature of crescentic glomerulonephritis. Endothelial cells express cell surface counter receptors for integrins to be adhered with neutrophils in circulating blood and glomerular

Correspondence and offprint requests to: Kazuo Suzuki PhD, Chief of Biodefense Laboratory, National Institute of Infectious Diseases (NIID-NIH), Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Email: ksuzuki@nih.go.jp

endothelial cells express intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). An elevated level of soluble ICAM-1 in sera of vasculitis patients has been reported [13–15]. Expression of the adhesion molecules on the surface of the endothelial cells is increased by stimulation with a wide variety of cytokines [16]. Similarly, elevated cytokine levels in the patient's blood seem to be a trigger for the enhancement of the expression of adhesion molecules on the surface, resulting in the development of vasculitis. However, a question remains whether ANCA also have direct effects on endothelial cells. Therefore, the direct effects of MPO-ANCA on the activation of glomerular endothelial cells must be elucidated in addition to the activation of neutrophils.

There are various studies available on the effects of sera or immunoglobulins from patients of autoimmune disease on endothelial cell activation. Johnson *et al.* [17] reported that autoantibody-positive serum samples from patients with vasculitis up-regulated ICAM-1 on human umbilical vein endothelial cells (HUVEC), although the molecular target of the autoantibody still remains unclear. De Bandt *et al.* [18] and Mayet *et al.* [19] demonstrated that anti-proteinase-3 antibodies from patients with Wegener's granulomatosis mediate ICAM-1 and VCAM-1 expression, respectively. Antibodies directed against endothelial cells from Scleroderma and Behçet's disease patients also activate HUVEC and up-regulated adhesion molecule expression [20,21]. However, the direct effect of MPO-ANCA on endothelial cells has not been investigated thus far. And, most research in this area has been done in HUVEC that can differ substantially from the cells in microvasculature, especially glomerular endothelial cells [22].

Here, we report the direct effect of polyclonal rabbit anti-recombinant mouse MPO antibody (anti-rmMPO) on expression of adhesion molecules in mouse glomerular endothelial cells (mGEC) and the mechanism of action.

Materials and methods

Primary culture of mouse glomerular endothelial cells and mesangial cells

We isolated mGEC from male C57BL/6 mice, which were maintained under specific pathogen-free conditions, according to the guidelines approved by the National Institute of Infectious Diseases Animal Care and Use Committee. Kidneys were obtained from freshly sacrificed mice perfused through the heart with cold Hank's balanced salt solution (HBSS) and glomeruli were prepared by a serial sieving method. Minced renal cortex tissue was serially passed through 150, 106 and 75 μm mesh stainless steel screens and glomeruli were collected using 53 μm mesh. The glomeruli, which contain small debris of renal tubule, were suspended in HBSS (Gibco-BRL Life Technologies, Gaithersburg, MD), washed twice by brief centrifugation (800 × *g*, 1 min) and digested in 1 mg/ml collagenase (Sigma, St Louis, MO) for 30 min at 37°C with occasional vortexing. Undigested glomeruli were pelleted by brief centrifugation and the supernatant containing single cell suspension of mGEC was suspended in growth medium [RPMI-1640 (Sigma) supplemented with 20% heat-inactivated foetal bovine serum (FBS, Gibco-BRL), 5 ng/ml vascular endothelial growth factor (VEGF) (Peprotech, Rocky Hill, NJ), 10 ng/ml bFGF (Sigma), 10 ng/ml epidermal growth factor (EGF) (Sigma), 20 U/ml heparin, 1 μg/ml hydrocortisone (Sigma), 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco-BRL)] and plated on fibronectin-coated 35 mm dishes (Becton Dickinson, Franklin Lakes, NJ). Small colonies of mGEC were observed within 1 week after plating. To remove contaminating mesangial and epithelial cells, brief trypsinization was performed until a culture with purity over 90% of endothelial cells was achieved. The cells were maintained in collagen-coated 100 mm culture dish (Iwaki, Tokyo, Japan).

Mesangial cells were isolated from the outgrowth of the undigested glomeruli according to the method described by Deocharan *et al.* [23] and maintained in RPMI-1640 supplemented with 20% heat-inactivated FBS, insulin-transferrin-selenium supplement (Gibco-BRL), 50 U/ml penicillin and 50 μg/ml streptomycin.

Rabbit anti-recombinant mouse myeloperoxidase antibody

Anti-rmMPO antibody was prepared as described previously [24]. Briefly, rmMPO was prepared from *Escherichia coli* transfected with a plasmid containing MPO cDNA of mouse origin (C57BL/6). The expressed recombinant protein consisted of His-tag-L-chain-H-chain of mouse MPO. Anti-rmMPO IgG was raised by immunization of rabbit with purified rmMPO and IgG fraction of the polyclonal antibody was isolated from the serum using protein A (Amersham Biosciences Co., Piscataway, NJ). The reactivity to purified native mouse MPO was confirmed as shown previously [24]. Normal rabbit IgG was prepared by the same procedure except for non-immunization with rmMPO.

Antibody titre to rmMPO was measured as described previously [24]. Briefly, rmMPO was coated onto an ELISA plate (Toyoshima Co., Tokyo, Japan) overnight at 4°C. The plate was blocked with 1% bovine serum albumin (BSA) (Sigma) and then incubated with anti-rmMPO IgG for 1.5 h at room temperature. The bound anti-rmMPO IgG was detected by 2 h incubation with alkaline phosphatase-labelled anti-rabbit IgG antibody (Bio-Rad, Hercules, CA). The bound secondary antibodies were subsequently quantified by changes in the absorbance at 405 nm after incubation with 1 mg/ml p-nitrophenyl phosphate (Sigma).

Modification of antibody

To deplete IgG from the anti-rmMPO antibody, 1 ml of anti-rmMPO IgG varying the concentration from 0 to 200 μg/ml was incubated with or without protein A Sepharose [100 μl slurry in phosphate-buffered saline (PBS), Amersham Biosciences Co.] for 1 h at 4°C. The resultant supernatants after centrifugation (3000 × *g*, 5 min) were used for experiments. To adsorb MPO-specific antibody, we have taken advantage of the insolubility of the rmMPO in the absence of a high concentration of urea and used it to remove specific binding antibodies from anti-rmMPO IgG by adsorption [25].