mecrosis factor-alpha and interleukin-1 beta by peripheral blood monocytes in the acute phase of Kawasaki disease. Eur. J. Pediatr. **1996**, *155*, 291–296.

23. Yoshioka, T.; Matsutani, T.; Iwagami, S.; Toyosaki-Maeda, T.; Yutsudo, T.; Tsuruta, Y.; Suzuki, H.; Uemura, S.; Takeuchi, T.; Koike, M.; Suzuki, R. Polyclonal expansion of TCRBV2- and TCRBV6-bearing T cells in patients with Kawasaki disease. Immunology **1999**, *96*, 465–472.
24. Hirano, J.; Hibi, S.; Andoh, T.; Ichimura, T. High levels of circulating interleukin-4 and interleukin-10 in Kawasaki disease. Int. Arch. Allergy Immunol. **1997**, *112*, 152–156.
25. Biezeveld, M.H.; Kuipers, I.M.; Geissler, J.; Lam, J.; Ottenkamp, J.J.; Hack, C.E.; Kuijpers, T.W. Association of mannose-binding lectin genotype with cardiovascular abnormalities in Kawasaki disease. Lancet **2003**, *361*, 1268–1270.
26. Khajoee, V.; Kariyazono, H.; Ohno, T.; Ihara, K.; Mizuno, Y.; Kusuhara, K.; Hara, T. Inducible and endothelial constitutive nitric oxide synthase gene polymorphisms in Kawasaki disease. Pediatr. Int. **2003**, *45*, 130–134.
27. Mallat, Z.; Besnard, S.; Duriez, M.; Deleuze, V.; Emmanuel, F.; Bureau, M.F.; Soubrier, F.; Esposito, B.; Deuz, H.; Fievet, C.; Staels, B.; Duverger, N.; Scherman, D.; Tedgui, A. Protective role of interleukin-10 in atherosclerosis. Circ. Res. **1999**, *85*, e17–e24.
28. Pinderski Oslund, L.J.; Hedrick, C.C.; Olvera, T.; Hagenbaugh, A.; Territo, M.; Berliner, J.A.; Fyfe, A.I. Interleukin-10 blocks atherosclerotic events in vitro and in vivo. Arterioscler. Thromb. Vasc. Biol. **1999**, *19*, 2847–2853.

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Contribution of Peroxidases in Host-Defense, Diseases and Cellular Functions

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SUMMARY: Peroxidases figure prominently in biology and contribute significantly to cell biology, host defense against infection, and pathogenesis of several inflammatory diseases. These varied and diverse aspects of peroxidase biochemistry and its clinical implications will be the subjects of in-depth analysis at the 4th International peroxidase meeting held in Kyoto. Specific topics range from the molecular basis of peroxidase structure and function to the clinical consequences of autoantibodies generated against myeloperoxidase (MPO), the peroxidase present in circulating neutrophils. Consideration of novel aspects of peroxidase biology, both unanticipated biochemical properties of MPO and the potential role of MPO in the pathogenesis of inflammatory diseases such as atherosclerosis, will also be included. In addition to peroxidases, the newly expanded family of NADPH oxidases will be discussed. We hope that this collection of scientists who share a common interest in peroxidase biology but each possess expertise in distinctly different aspects of the subject will provide a setting for spirited discussion and a lively exchange of views to yield advances in understanding and to create new applications of those insights to benefit clinical medicine, agriculture and industry.

1. Peroxidase and related oxidase studies

In 2000, a book entitled "The peroxidase multigene family of enzymes" (1) updated the peroxidase field and summarized the proceedings of the 2nd International Peroxidases Meeting held in Chiemsee and organized by Drs. Petro Petrides and William Nauseef. However since that time, several advances in the peroxidase field have occurred. Recent work has uncovered novel biochemistry, new gene families, and knock-out animals have been used to address important and unanswered questions. We convene now in Kyoto peroxidase scientists from around the world to discuss ongoing studies and share new insights into the biology of this important protein family.

Myeloperoxidase (MPO): The organizing principle of the first peroxidase meeting was an interest in myeloperoxidase, the family member expressed in exclusively in cells of a neutrophilic or monocytic lineage. As neutrophils are the dominant cellular component of the human innate immune system and the oxygen-dependent antimicrobial system of neutrophils is the most efficient defense against microbes, MPO has a central place in neutrophil microbicidal action. Unique among the animal peroxidase family, MPO catalyzes the two electron oxidation of chloride ion in the presence of hydrogen peroxide to generate hypochlorous acid, a potent antimicrobial agent. The MPO-hydrogen peroxide-chloride system is responsible for microbicidal activity against a wide range of organisms and has served as the paradigm for neutrophil oxidative killing of bacteria.

Dysfunction of host-defense due to MPO-deficiency in human: Despite its central role in normal host defense, the phenotype of inherited deficiency of MPO has not been clearly demonstrated as increased risk for infectious complications. Four allelic mutations resulting in inherited MPO deficiency have been previously reported (2-5) R569W, Y173C and M251T and G501S. The defect mechanisms and manner of inheritance has been described in detail (3). The prevalence of complete MPO deficiency in Japan is estimated to be 1.75/100,000, a value 14- to 28-fold lower than that of the United States and Europe, respectively (1,6). The molecular basis of deficiencies in Japan and their relation to the genotypes seen elsewhere are the subject of ongoing study.

MPO-deficient mice: Whereas population studies on the prevalence of complications among human with inherited MPO deficiency have been of limited use, the application of molecular techniques to generate mice deficient in MPO has proven a useful experimental tool. The earlier reports of the clinical consequences of MPO deficiency described the increased risk in affected individuals for disseminated and often fatal candidiasis. This association between MPO activity and host defense against Candida was recapitulated in the mouse model by Aratani et al. (7). The availability of the MPO knock-out mouse also makes possible testing novel hypotheses regarding the role of MPO in pathogenesis of diseases unrelated to infection. Unexpectedly MPO-deficient mice show an increase in experimentally induced atherosclerosis (8), perhaps highlighting important species differences between mouse and man. Nonetheless, the mouse model will provide important and novel insights into MPO biology.

Peroxidase related to Disease Activity: MPO-ANCA related diseases: In addition to host-defense function, MPO is also a target molecule of MPO-specific anti-neutrophil cytoplasmic autoantibody (MPO-ANCA). Antibodies directed against cytoplasmic constituents of the neutrophil, specifically MPO and proteinase 3, have been extensively described as markers for systemic vasculitis and crescentic glomerulonephritis. Evidence further indicates that MPO and the MPO-ANCA are risk factors for the development of immune-mediated renal disease, as the sera of patients with microscopic polyangiitis and crescentic glomerulonephritis (CrGN), high titers of MPO-ANCA are frequently detected. Studies in the mouse model promise to provide important insights into the pathogenesis of this vasculitic disease.

Immunomodulatory therapy for MPO-ANCA related diseases: Many therapeutic trials for MPO-ANCA related diseases have been performed, especially those for rapidly progressive glomerulonephritis (RPGN) due to CrGN. Among the various interventions tested, intravenous immunoglobulin (IVIg) has improved the outcome of this highly life-threatening disease in Europe. Although there are many potential mechanisms underlying the beneficial effect of IVIg, one may be the suppression of the presentation of MPO to stimulated neutrophils. In addition other immunomodulatory effects including the correction of abnormally deviated Th1/Th2 balance and suppression of the highly elevated cytokine activity may play a role (submitted). The favorable outcome of the IVIg for MPO-ANCA related RPGN in Japan and the partial elucidation of the mechanism of action will be presented.

NOX family: MPO action requires hydrogen peroxide and in stimulated neutrophils, the NADPH oxidase generates reactive oxygen species, including hydrogen peroxide, from molecular oxygen. The phagocyte NADPH oxidase is a multicomponent enzyme containing membrane and cytosolic components that assemble at the membrane when neutrophils are stimulated by an appropriate agonist. The membrane component of the NADPH oxidase is a heterodimeric protein composed of gp91*phox* and p22*phox*. Recently homologues of gp91*phox* have been described, giving birth to the NOX (NADPH oxidase) protein family. Previously work from the laboratories of Krause and of Sumimoto were presented at 6th MPO meeting at Atami in 2000 (Abstract Book). As the family grows and new data emerge, it seems that the NOX enzymes have two physiological functions: 1) Host defense, typified by the phagocyte NADPH oxidase and indirectly suggested for NOX1, DUOX1, and DUOX2.

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and 2) Biosynthetic processes, as seen with for DUOX enzymes, implicated in biosynthesis of thyroid hormone in mammals and in the crosslinking of extracellular matrix in *C. elegans*. In addition, NOX enzymes are involved in signaling function of ROS and new information will be presented at the meeting.

2. Role of the International Peroxidase Meeting

Drs. Suzuki and Nauseef have organized this international meeting to extend insights into role of MPO and other peroxidases, as originally intended at the first peroxidase meeting. The first meeting on myeloperoxidase was inspired and organized by Dr. Dolphe Kutter and held in Luxemburg in 1996. It was a small meeting but served to confirm the need for an international meeting where investigators, clinical and basic, who shared an interest in the biology of MPO could come together to discuss important aspects of its biology and role in health and disease. The second meeting was convened to meet this charge and was held in a Benedictine Abbey on Fraueninsel in Lake Chiemsee, in Bavaria, Germany. Organized by Petro Petrides from Munich, Germany and William Nauseef from the University of Iowa, USA, the meeting in 1998 was a great success, generating the publication of a book "The peroxidase multigene family of enzymes: Biochemical basis and clinical applications" and setting the stage for future meetings. The 3rd conference was held in Vienna, Austria in 2002 and was organized by Christian Obinger. Christian expanded the chemistry component of the meeting and expanded the format to include not only other animal peroxidases but also peroxidases from the plant world.

The 4th International Peroxidase Meeting: The 4th International Peroxidase Meeting is held on October 27-30, 2004, Kyoto Palulu Plaza (www.nih.go.jp/MPO/). Based on the background of International Peroxidase Meeting, we will organize the 4th International Peroxidase Meeting joined with the 10th MPO meeting organized by Muso, Kitano Hospital. The MPO meeting has been held in Japan since 1995, making this the 10th anniversary MPO Meeting. Thus it seems appropriate celebrate this special milestone by joining with the 4th International Peroxidase Meeting. The program for the meeting has been organized around the following format: Opening Lecture: Contribution of MPO in vasculitis development by K. Suzuki, and Plenary Lecture: Lessons from MPO deficiency about functionally important structural features by W. Nauseef will be announced. Special lectures-1. Clinical treatment for patients with MPO-ANCA by D. Jayne, E. Muso, and Y. Aratani, and -2. New aspects of peroxidases and oxidases: Nox/Duox family NADPH oxidases: expression patterns and possible physiological functions by K-H Krause will be presented. In addition, five sessions: MPO-ANCA-related diseases, action and molecular aspects of peroxidases, inflammation and peroxidaserelated diseases, peroxidases and NADPH oxidases, and reaction of MPO will be joined with poster presentations. Conferees registered are from Austria, France, Germany, Italy, New Zealand, Russia, Spain, Sweden, Switzerland, UK, and USA in addition to Japan.

Thus, we will have presentations of various peroxidases and other oxidases in this meeting. We intend to provide a venue at these sessions for discussion of all aspects of peroxidase biology. Finally, we hope that the insights and information provided at the meeting will reveal new roles for the peroxidases and other oxidases in health and disease.

The next meeting: The 5th meeting will be held in Christchurch in New Zealand and organized by Dr. Tony Kettle in The Christchurch Medical School.

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REFERENCES

1. Petrides, P. E. and Nauseef, W. M. (eds.) (2000): The Peroxidase Multigene Family of Enzymes. Springer-Verlag, Berlin.
2. Nauseef, W. M., Brigham, S. and Cogley, M. (1994): Hereditary myeloperoxidase deficiency due to a missense mutation of arginine, 569, to tryptophan. J. Biol. Chem., 269, 1212-1216.
3. DeLeo, F. R., Goedken, M., McCormick, S. J. and Nauseef, W. M. (1998): A novel form of hereditary myeloperoxidase deficiency linked to endoplasmic reticulum/proteasome degradation. J. Clin. Invest., 101, 2900-2909.
4. Romano, M., Dri, P., Dadalt, L., Patriarca, P. and Baralle, F. E. (1997): Biochemical and molecular characterization of hereditary myeloproliferative deficiency. Blood, 90, 4126-4134.
5. Ohashi, Y.Y., Kameoka, Y., Persad, A.S., Kohi, F., Yamagoe, S., Hashimoto, K. and Suzuki, K. (2004): Novel missense mutation found in Japanese patient with myeloperoxidase deficiency. Gene, 327, 195-200.
6. Nunoi, H., Kohi, F., Kajiwara, H. and Suzuki, K. (2003): Prevalence of inherited myeloperoxidase deficiency in Japan. Microbiol. Immunol., 47, 527-531.
7. Aratani, Y., Koyama, H., Nyui, S., Suzuki, K., Kura, F. and Maeda, N. (1999): Severe impairment in early host defense against *Candida albicans* in mice deficient in myeloperoxidase. Infect. Immun., 67, 1828-1836.
8. Brennan, M. L., Anderson, M. M., Shih, D. M., Qu XD, Wang X., Mehta, A. C., Lim, L. L, Shi, W., Hazen, S. L., Jacob, J. S., Crowley, J. R., Heinecke, J. W. and Lusis, A. J. (2001): Increased atherosclerosis in myeloperoxidase-deficient mice. J. Clin. Invest., 107, 419-430.

Contribution of Myeloperoxidase in Vasculitis Development

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SUMMARY: Infiltrated neutrophils is believed to contribute to the progression of vasculitis. In particular, myeloperoxidase (MPO)-specific antibodies against neutrophils, anti-neutrophil cytoplasmic antibodies (MPO-ANCA) is involved in the development of vasculitis microscopic polyangiitis etc. In Japan a higher percentage of MPO-ANCA than that in Europe has been reported In addition, we showed a correlation of MPO-ANCA epitopes of Kawasaki disease patients by 47% with that of mothers'. On the other hand, mice having CADS/CAWS-induced vasculitis is a good model for the analysis of the production of MPO-ANCA. We have clarified that MPO is a major antigen for MPO-ANCA production using MPO KO mice. We also investigated the role of activated neutrophils in nephritis renal lesions using SCG/Kj mice. In the phase of nephritis with low grade of proteinuria, the spontaneous release of MPO from peripheral neutrophils increased, indicating that neutrophils are activated and contribute to the development of active crescentic lesion in SCG/Kj mice.

Activated neutrophils in patients with vasculitis suggest that they contribute to the progression of vasculitis has been investigated (1). Target molecules of the antibodies against neutrophils, anti-neutrophil cytoplasmic antibodies (ANCA) related to the develop-

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ment of vasculitis are myeloperoxidase (MPO) and proteinase-3 (PR3) contained in the granules of neutrophils. In particular, MPO-ANCA is associated with certain subtypes of primary vasculitis. Thus, MPO-ANCA has been demonstrated to be involved in the development of vasculitis microscopic polyangiitis etc. (2). Patients with MPO-ANCA related glomerulonepheritis (GN) also show an increase in the activated neutrophils in peripheral blood (1) in addition to Kawasaki disease. In Japan a higher percentage of MPO-ANCA than that in Europe has been reported (3). Recently, role of ANCA by the European Vasculitis Study Group trials have also been studied (4).

Furthermore, in addition to these diseases, elevation in the levels of MPO-ANCA in sera of patients with Kawasaki disease and systemic lupus erythematosus (SLE) has also been observed. Then, we analyzed a correlation of MPO-ANCA epitopes of Kawasaki disease patients with their mother to know the etiology related to MPO-ANCA. Most of healthy mothers showed MPO-ANCA positive in their sera with lower titer. Epitopes in sera of patients were coincident by 47% with that of mothers', but less father's (Table 1), suggesting that source of auto-antibody MPO-ANCA may be same to that of patient's mother (5).

Table 1. Correlation of epitopes of MPO-ANCA of KD patients with their parents

MPO-ANCA Positive 85.7 %		
Correlation	Epitopes	% Prevalence
with Father	Ha	5.9
	Hg	5.9
	No-relation	0
with Mother	Ha	17.6
	Hg	29.4
	No-relation	11.8
with parents		5.9

Eighteen families were examined in 42 patients in Hiroshima City Hospital from Mar. 1998 to Dec. 2000.
Ha: N-terminus of heavy chain, Hg: C-terminus.

On the other hand, ANCA may be important in the pathophysiology of necrotizing vasculitis due to neutrophils activated with inflammatory cytokines such as tumor necrosis factor- α (TNF-α), IL-6 and IL-8 in blood circulation. Interestingly, it has been demonstrated that ANCA activates neutrophils primed with TNF-α in vitro, resulting in the translocation of ANCA antigens to the cell surface.

As the basis for clinical studies, animal models are often used to understand the mechanisms of the development of vasculitis, and to establish therapeutic strategies. Both MRL lpr/lpr, and SCG/Kj strains are known to show high levels of MPO-ANCA in association with renal lesions including GN and vasculitis. On the other hand, CADS or CAWS-induced vasculitis have been used for the analysis of the development and progression of vasculitis (6). CADS/CAWS-induced vasculitis with coronary arteritis is a good model for the analysis of the production of MPO-ANCA. We have clarified that MPO is a major antigen for MPO-ANCA production using MPO KO mice (7). Moreover, the study using NZB/W F1 mice with the Fcγ receptor-deficiency has shown that Fcγ receptor on neutrophils and/or macrophages has been demonstrated to be necessary in the occurrence of GN. However, the more precise pathogenic roles of MPO-ANCA and neutrophils in the development of GN and vasculitis in these murine models are undetermined. We investigated the role of activated neutrophils in nephritis renal lesions using SCG/Kj mice. The mice having spontaneous CrGN and vasculitis showed higher levels of MPO-ANCA and TNF-α than those of normal mice C57BL/6. In the phase of nephritis with low grade of proteinuria, the spontaneous release of MPO from peripheral neutrophils increased, while superoxide generation increased before spontaneous MPO release occurred. In addition, the renal lesion in histological observations aggravated with aging and the glomerular neutrophil infiltration was positively correlated with MPO-ANCA levels as well as with histological indices of nephritis, active renal injury score, especially crescent formation was correlated with spontaneous MPO release. These findings indicate that neutrophils are activated and contribute to the development of active crescentic lesion in SCG/Kj mice (8).

The certain neutrophil infiltration into tissue showing vasculitis suggests that neutrophils may cause the development of vasculitis. MPO released from activated neutrophils occasionally causes self-damage to tissues due to the toxicity of its product OCl^- or other radicals such as O_2^-, H_2O_2, OCl^-, NO as well killing fungi improved with MPO-KO mice.

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REFERENCES

1. Minoshima, S., Arimura, Y., Nakabayashi, K., Kitamoto, K., Nagasawa, T., Ishida-Okawara, A. and Suzuki, K. (1997): Increased release of myeloperoxidase in vitro from neutrophils of patients with myeloperoxidase-specific anti-neutrophil cytoplasmic antibody (MPO-ANCA) related glomerulonephritis. Nephrology, 3, 527-534.
2. Savage, C. O., Winearls, C. G., Jones, S. J., Marshall, P. D. and Lockwood, C. M. (1987): Prospective study of radioimmuno-assay for antibodies against neutrophil cytoplasm in diagnosis of systemic vasculitis. Lancet, 1, 1389-1393.
3. Yashiro, M., Muso, E., Itoh-Ihara, T. et al. (2000): Significant high regional morbidity of MPO-ANCA-related angitis and/or nephritis with respiratory tract involvement after the 1995 great earthquake in Kobe (Japan). Am. J. Kidney. Dis., 35, 889-895.
4. Jayne, D. (2001): Update on the european vasculitis study group trials. Curr. Opin. Rheumatol., 13, 48-55.
5. Suzuki, K. and Okazaki, T. (2001): Correlation of MPO-ANCA epitope between patients with Kawasaki disease and their mothers.The 7th International Kawasaki Disease Symposium. Abstracts, p. 58.
6. Miura, N. N., Shingo, Y., Adachi, Y., Ishida-Okawara, A., Oharaaseki, T., Takahashi, K., Naoe, S., Suzuki, K. and Ohno, N. (2004): Induction of coronary arteritis with administration of CAWS (*Candida albicans* Water-Soluble fraction) depending on mouse strains. Immunopharmacol. Immunotoxicol. (in press).
7. Ishida-Okawara, A., Oharaseki, T., Takahashi, K. et al. (2001): Contribution of myeloperoxidase to coronary artery vasculitis associated with MPO-ANCA production. Inflammation, 25, 381-387.
8. Ishida-Okawara, A., Ito-Ihara, T., Muso, E., Ono, T., Saiga, K., Nemoto, K. and Suzuki, K. (2004): Neutrophil contribution to the crescentic glomerulonephritis in SCG/Kj mice. Nephrol. Dial. Transplant., 19, 1708-1715.

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Genomic Variations in Myeloperoxidase Gene in the Japanese Population

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SUMMARY: Myeloperoxidase (MPO; EC 1.11.1.7) is a lysosomal hemeprotein that plays an important role in the host defense mechanism against microbial diseases. This neutrophil disorder, characterized by the lack of MPO, may result in a weakened defense activity. Complete MPO deficiency has been postulated to be to originate from genomic mutation. Recently, two Japanese patients were reported with MPO deficiency. Both had base substitutions in the exon 9 region of the MPO gene; a region in close proximity functionally important residue, His502. Genomic DNA from 387 Japanese individuals was examined to determine the prevalence of these recently discovered base substitutions. None of these DNA samples possessed the mutations found in the MPO deficient cases, though two synonymous and one non-synonymous mutation were found. The frequency of mutation in the exon 9 coding region was estimated to be one heterozygote in 129, thus the homozygote of such mutations would be revealed one in 16,000 in the Japanese population.

Myeloperoxidase (MPO) is a lysosomal hemeprotein located in azurophilic granules of neutrophils and monocytes. MPO is part of the host defense system and is responsible for microbicidal activity against a wide range of organisms. A deficiency in MPO is speculated to be associated with a decreased level of immunity (4). Aratani et al. (1) has described the association with this deficiency and continuous infection of *Candida albicans* in MPO knock-out mice.

In the human population, the prevalence of complete MPO deficiency in Japan is estimated to be 1.75/100,000, a value 14- to 28-fold lower than that of the United States and Europe, respectively (8). Three allelic mutations related to MPO deficiency have been previously reported: R569W (5), Y173C (3), and M251T (7). MPO research is now making headway with the genetic analysis of patients with complete and partial MPO deficiency.

Research conducted over the past year entailed identifying mutations found in cases afflicted with MPO deficiency and estimating the prevalence of these mutations in a control cohort. Two novel non-synonymous mutations were researched during this time period; a glycine to serine substitution (G501S) and an arginine to cysteine substitution (R499C), both found on the exon 9 region of the MPO gene. The G501S mutation, first reported in the Japanese population, (6) was found originally in a patient with complete MPO deficiency. Neutrophil function analysis revealed that MPO activity was significantly diminished with slightly elevated superoxide production.

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Another patient was later identified with complete MPO deficiency. As with the first case, MPO activity was diminished with increased superoxide production. In this case, a new mutation was also found in the region responsible for coding MPO: a point mutation in exon 9 region that resulted in an arginine to cysteine substitution (R499C) (Persad, in preparation). Primer sets used in the recognition of mutations found in both patients are described in (6).

A total of 387 DNA samples served as a comparison cohort in the investigation of a possible link between these identified mutations and the presence of MPO deficiency. Due to difficulties in obtaining samples from a large number of healthy individuals, the control group used consisted of DNA from rheumatoid arthritis samples (21%), hepatitis C samples (41%) and healthy blood donors (38%), none of which had information on levels of MPO activity or superoxide production. Among these samples, three isolated point mutations were found in exon 9, all of which were heterozygous, with two of the mutations being synonymous in nature (1434 G/A, 1478 C/A; the numbers indicate the base position from Adenine of first ATG in mRNA). The third isolated mutation (1464 T/C) would result in an amino acid substitution from isoleucine to threonine. This mutation has not been confirm nor is MPO activity available for this DNA sample. All 387 samples did not possess the non-synonymous mutations found in the MPO deficient cases, thus drawing a more defined postulation that G501S and R499C may be associated with complete MPO deficiency.

Complete MPO deficiency is hereditary and requires the possession of two recessive alleles. This knowledge, coupled with population dynamics in Japan, produced a scenario in which cases with this type of deficiency can serve as sentinels in the detection of clusters of individuals that are heterozygous for these novel mutations.

Both novel mutations, G501S and R499C, have thus far been only found Japanese individuals. An interesting phenomenon, unlike previously identified mutations, is the proximity of these mutations to each other as well as to the histidine at codon 502 that is pivitol to heme binding (2). Based on this research, it is speculated that mechanism of action of these mutations to induce MPO deficiency is via the interruption of heme binding due to the amino acid substitution caused.

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REFERENCES

1. Aratani, Y., Kura, F., Watanabe, H., Akagawa, H., Takano, Y., Suzuki, K., Maeda, N. and Koyama, H. (2000): Differential host susceptibility to pulmonary infections with bacteria and fungi in mice deficient in myeloperoxidase. J. Infect. Dis., 182, 1276-1279.
2. Davey, C. A. and Fenna, R. E (1996): 2.3 A resolution X-ray crystal structure of the bisubstrate analogue inhibitor salicylhydroxamic acid bound to human myeloperoxidase: a model for a prereaction complex with hydrogen peroxide. Biochemistry, 35, 10967-10973.
3. DeLeo, F. R., Goedken, M., McCormick, S. J. and Nauseef, W. M. (1998): A novel form of hereditary myeloperoxidase deficiency linked to endoplasmic reticulum/proteasome degradation. J. Clin. Invest., 101, 2900-2909.
4. Lanza, F. (1998): Clinical manifestation of myeloperoxidase deficiency. J. Mol. Med., 76, 676-681.
5. Nauseef, W. M., Cogley, M. and McCormick, S. (1996): Effect of the R569W missense mutation on the biosynthesis of myeloperoxidase. J. Biol. Chem., 271, 9546-9549.
6. Ohashi ,Y. Y., Kameoka, Y., Persad, A. S., Koi, F., Yamagoe, S., Hashimoto, K. and Suzuki, K. (2004): Novel missense mutation found in a Japanese patient with myeloperoxidase deficiency. Gene, 327, 195-200.
7. Romano, M., Dri, P., Dadalt, L., Patriarca, P. and Baralle, F. E (1997): Biochemical and molecular characterization of hereditary myeloproliferative deficiency. Blood, 90, 4126-4134.
8. Nunoi, H., Kohi, F., Kajiwara, H. and Suzuki, K. (2003): Prevalence of inherited myeloperoxidase deficiency in Japan. Microbiol. Immunol., 47, 527-531.

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In Vivo Role of Myeloperoxidase for the Host Defense

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SUMMARY: Myeloperoxidase (MPO) is located within neutrophils capable of producing HOCl. To define the in vivo role of MPO, we have generated MPO-knockout (MPO-KO) mice. The mice without MPO developed normally. However, MPO-KO mice showed severely reduced cytotoxicity to various microorganisms such as *Candida albicans*, *Aspergillus fumigatus*, and *Klebsiella pneumoniae*, demonstrating that MPO-dependent oxidative system is important for host defense against fungi and bacteria, although the effect varies from species to species of pathogens. To compare the importance of MPO and NADPH-oxidase for host defense, MPO-KO and chronic granulomatous disease (CGD) mice were infected with different doses of *C. albicans*, and their infection severity was analyzed. CGD mice exhibited increased mortality and tissue fungal burden in a dose-dependent manner, whereas normal mice showed no symptoms. Interestingly, at the highest dose, the mortality of MPO-KO mice was comparable to CGD mice, but was the same as normal mice at the lowest dose. These results suggest that MPO and NADPH-oxidase are equally important for early host defense against a large inocula of *Candida*.

Neutrophils are believed to be the first line of defense against invading microorganisms, but in vivo roles of reactive oxygens produced by neutrophils are not well known. Myeloperoxidase (MPO) catalyzes reaction of hydrogen peroxide with chloride ion to produce hypochlorous acid that is used for microbial killing by phagocytic cells. To define the in vivo role of MPO, we have generated mice having no peroxidase activity in their neutrophils and monocytes (1). MPO-deficient (MPO-KO) mice showed severely reduced cytotoxicity to *Candida albicans*, *Aspergillus fumigatus*, *Trichosporon asahii*, and *Pseudomonas aeruginosa*, and others (Table 1) (1-3), demonstrating that MPO-dependent oxidative system is important for host defense against fungi and bacteria.

However, the significance of MPO compared to the NADPH-oxidase is still unclear because individuals with MPO deficiency are usually healthy in contrast to the patients with chronic granulomatous disease (CGD) who present clinical symptoms early in life and die with recurrent infections during childhood. To better understand the contributions of MPO and NADPH-oxidase to antifungal defense mechanisms, we compared the susceptibility of MPO-KO mice and CGD mice to the infections with *C. albicans*. Interestingly, at the highest dose, the mortality of MPO-KO mice was comparable to CGD mice, but was the same as normal mice at the lowest dose (Fig. 1). At the middle dose, the number of fungi disseminated into various organs of the MPO-KO mice was comparable to the CGD mice in one week after infection, but it was significantly lower in 2 weeks (4). These results suggest that MPO and NADPH-oxidase are equally important for early host defense against a large inocula of *Candida*.

Table 1. Recovery of fungi and bacteria from the lungs of wild-type and MPO-KO mice after intranasal inoculation

Organism	log CFU/lung			B/A
	0.5 h	48 h		
		(A) Wild type	(B) Mutant	
Candida albicans	6.7	4.8 ± 0.2	6.3 ± 0.2	30.1
	5.7	3.6 ± 0.2	5.4 ± 0.1	66.1
Cadida tropicalis	6.0	4.1 ± 0.2	5.6 ± 0.1	33.1
	5.1	3.0 ± 0.2	3.5 ± 0.1	3.0
Trichosporon asahii	6.0	4.7 ± 0.1	6.1 ± 0.1	26.3
	5.1	3.6 ± 0.2	4.2 ± 0.1	3.9
Aspergillus fumigatus	5.7	2.2 ± 0.2	3.6 ± 0.2	22.9
	5.2	1.8 ± 0.5	2.9 ± 0.2	12.9
Pseudomonas aeruginosa	5.8	3.5 ± 0.3	6.3 ± 0.3	550.0
	5.0	2.8 ± 0.1	2.9 ± 0.2	1.3
Klebsiella pneumoniae	6.8	3.3 ± 0.3	4.3 ± 0.3	9.3
	5.2	<1.0	1.9 ± 0.9	>8.1

CFU of inoculated fungi and bacteria in mouse lungs was assessed at indicated times.

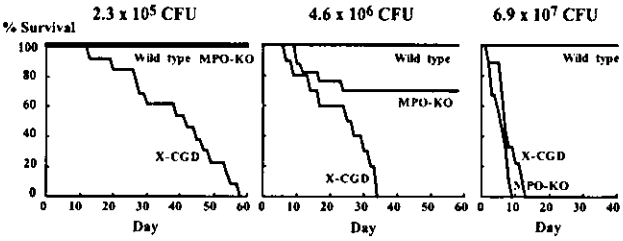

Fig. 1. Survival of mice after *C. albicans* infection. Wild-type, MPO-KO, and X-CGD mice were intraperitoneally infected with the indicated doses of *Candida*.

Hereditary MPO deficiency is a common neutrophil defect with estimated incidence of 1 in 2,000 in the United States, and of 1 in 50,000 in Japan. Our present results suggest that MPO-deficient individuals could exhibit similar problems as CGD patients if exposed to a large amount of microorganisms.

ACKNOWLEDGMENTS

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REFERENCES

1. Aratani, Y., Koyama, H., Nyui, S., Suzuki, K., Kura, F. and Maeda, N. (1999): Severe impairment in early host defense against *Candida albicans* in mice deficient in myeloperoxidase. Infect. Immun., 67, 1828-1836.
2. Aratani, Y., Kura, F., Watanabe, H., Akagawa, H., Takano, Y., Suzuki, K., Maeda, N. and Koyama, H. (2000): Differential host susceptibility to pulmonary infection with bacteria and fungi in mice deficient in myeloperoxidase. J. Infect. Dis., 182, 1276-1279.
3. Aratani, Y., Kura, F., Watanabe, H., Akagawa, H., Takano, Y., Suzuki, K., Dinauer, M. C., Maeda, N. and Koyama, H. (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, 1557-1563.
4. Aratani, Y., Kura, F., Watanabe, H., Akagawa, H., Takano, Y., Suzuki, K., Dinauer, M. C., Maeda, N. and Koyama, H. (2002): Critical role of myeloperoxidase and nicotineamide adenine dinucleotide phosphate-oxidase in high-burden systemic infection of mice with *Candida albicans*. J. Infect. Dis., 185, 1833-1837.

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Intravenous Immunoglobulin (IVIg) Therapy in MPO-ANCA Related Polyangiitis with Rapidly Progressive Glomerulonephritis in Japan

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SUMMARY: For 30 myeloperoxidase (MPO) antineutrophil cytoplasmic antibody (ANCA) related rapidly progressive glomerulonephritis patients (male 17, female 13 , average age of 68 ± 11.8 years old), intravenous immunoglobulin (IVIg) (400 mg/kg/day) was administered for 5 consecutive days before or along with conventional immunosuppressive therapy in Japan. Twenty patients were treated with IVIg before the start or newly increase of conventional therapy and evaluated the independent effect of this therapy. In these patients, just after IVIg, significant decrease of CRP from 8.61 ± 5.77 to 5.47 ± 4.50 mg/dl ($P < 0.001$) was noted with improvement of elevated serum creatinine in 12 out of 19 patients (63%). In the analysis of the overall outcome of 30 patients, at 3 months after IVIg and following conventional therapy, no patients showed renal death except 4 for whom hemodialysis had been started before IVIg. At 6 months, renal survival rate were 92% (newly renal death 2 out of 26) and 2 patients died due to cerebral bleeding (survival rate was 93%). No fatal infection was noted. IVIg might be the potent inducible therapy which can be promoted before the beginning of conventional immunosuppressant treatment for relatively aged and lower immunopotent MPO-ANCA patients in Japan.

Rapidly progressive glomerulonephritis (RPGN) is often associated with systemic vasculitis presenting antineutrophil cytoplasmic antibody (ANCA) (1). These ANCA-related RPGN often necessitated aggressive immunosuppressive treatment using high dose corticosteroid and cyclophosphamide (CYC) which sometimes brought about severe side effects especially sometime fatal infections, since these diseases often occur in relatively aged populations. To avoid these fatal side effects, intravenous immunoglobulin (IVIg) therapy has been utilized in Europe for these ACNA-related vasculitis and has been proved to be clinically safe, suppress disease activity for at least 1 year, and reduce the total dose of immunosuppressive agents (2-4). Although these reports of this therapy are useful, it is necessary to be prudent for the direct application of these results for those in Japan because in Europe, the distribution of the type of disease tends to orient to Wegener's granulomatosis (WG) which are not so frequently experienced as the causative disease of RPGN in Japan. Recent survey for the incidence of RPGN in Japan revealed that 62% of 593 RPGN patients from 1996 to 2000 were MPO-ANCA positive, in contrast to only 4% of PR3-ANCA positive patients (5). Therefore, the independent survey is necessary to prove the efficacy of this therapy as the safe and potent induction therapy for MPO-ANCA related RPGN.

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Patients and methods: **Patients:** Thirty MPO-ANCA related RPGN patients (male 17, female 13) in Japan were treated with IVIg before or during the conventional immunosuppressant therapy using corticosteroid and CYC. These patients were treated in 5 hospitals (Kitano Hospital: 12 cases, Ehime Prefecture Hospital: 8, Osaka University Hospital: 4, Tsukuba University Hospital: 4 Kyoto University Hospital: 4) in Japan separately from 2001 to 2003. Average age of the patients were 68 ± 11.8 from 36 to 83 years old. All patients showed elevated serum MPO-ANCA as well as characteristic pathology observed in the renal biopsy specimen. All patients were provided written informed consent for renal biopsy and the present treatment protocol.

Treatment protocol: For all patients except one, IVIg was administered intravenously once for 5 consecutive days (400 mg/kg/day) (Kenketus Venilon-I, Teijin Co., Ltd., Tokyo, or Kenketsu Glovenin-I, Nihon Pharmaceutical Co., Ltd., Tokyo, Japan). One patient was treated with IVIg twice during her hospital course. Twenty patients (male 12, female 8, average age: 71.3 ± 8.82) were treated with IVIg before the start or newly increase of conventional immunosuppressive therapy and the effect of the IVIg could be evaluated independently.

Clinical features before IVIg treatment: All patients showed elevation of creatinine (Cre) before IVIg with mean value of 4.04 ± 2.94 mg. Twenty-one of them were diagnosed RPGN with rapid increase of Cre more than double within 3 months before entry. For four patients, hemodialysis had to be started before IVIg therapy. The activity of the inflammation were severe with the mean CRP of 7.2 ± 5.5 mg/dl. All patients were MPO-ANCA positive with 243.7 ± 355.2 EU.

These patients were under various complicated diseases prior to the burst of MPO-ANCA disease. Ten cases showed one or more pulmonary diseases such as pulmonary fibrosis: 7 cases, latent tuberculosis: 2, aspergilosis: 1, other bacterial infections: 3. Other complicasions were as follows: Idiopathic thrombocytic purpura 3, hepatitis B virus carrier 2, diabetes mellitus 2, aortic aneurysm 1, mononeuropathy 1, malignancy 1 (laryngeal cancer). In addition, 2 patients were MRSA carrier.

RESULTS

Response to the IVIg therapy: For 20 patients independently treated with IVIg before the start or the increase of the immunosuppressants, the evaluation of the response to this therapy was performed separately within 14 days for 19 out of 20 patients whose data just after the IVIg were available. The significant decrease of the CRP was noted just after the IVIg from 8.61 ± 5.77 to 5.47 ± 4.50 mg/dl ($P < 0.001$). Although the average level of Cre did not show a significant decrease within such short period of observation (from 3.46 ± 2.34 to 3.39 ± 2.16 mg/dl P:n.s.), it was noteworthy that the elevation of Cre before IVIg stopped in one and rather decreased in 12 patients.

The evaluation of the effect of IVIg on MPO-ANCA titers was available in 13 of 19 patients. There was no significant decrease of these titers just after the IVIg (from 253.30 ± 275.40 to 410.07 ± 621.56 EU).

Outcome of the patients: Following or along with IVIg, patients were treated with conventional immunosuppressants including corticosteroid. Two out of 30 patients did not need to add additional therapy after IVIg. For other 28 patients, average initial dose of 33.4 ± 11.2 mg of Prednisolone (0.6 ± 0.1 mg/kg/day) were administered. For those without complicated infectious diseases, pulse therapy of methylprednisolone (0.5-1 g/day for 3 days) were performed in 8 and oral CYC 50 mg/day for 9 patients for their severely active state of the disease. Plasma exchange was also performed for 2 patients. After 3 months of these treatments, activity of the disease was completely suppressed with average CRP value of 0.80 ± 2.44 mg/dl ($P < 0.001$ v.s. before IVIg). The elevated Cre was also significantly suppressed to 2.20 ± 1.20 mg/dl ($P < 0.01$) and 25 out of 30 patients showed the improvement of renal function. Significant decrease of the MPO-ANCA titers were also noted with the mean value of 41.44 ± 81.42 EU ($P < 0.001$). In 12 patients, ANCA were completely negative at this point. As for the overall outcome of renal function, except four patients who were started hemodialysis before treatment, no renal death was noted at 3 months, in 1 at 6 months (overall renal survival rate: 77%, and 92% except for those who were hemodialyzed before IVIg) and in another one patient after 6 months until the end of year 2003. As for the life survival, before 6 months 2 patients (survival rate 93%) and more 3 patients died after 6 months. The causes of death were cerebral bleedings for two and malignancies such as malignant lymphoma for 2 and one gastric cancer after 6 months following IVIg. It should be noted that there was no fatal infection in all IVIg treated patients.

DISCUSSION

Recently we have experienced the favorable results of IVIg monotherapy for 15 RPGN patients showing rapid decrease of CRP, WBC and ANCA titers in association with down regulation of the serum inflammatory cytokines especially of TNF α (Ito-Ihara, in submission). In the current survey, a significant anti-inflammatory effect of IVIg therapy was also proved even though the increase of the samples. In Japan survey of RPGN, 6 months renal survival rate was 70% and survival rate was 74% in MPO-ANCA positive RPGN with conventional immunosuppressant therapy (5). Comparing with these results, the 92% of renal survival rate and especially 93% life survival were remarkably high. In particular, the absence of the death due to fatal infection even following usage of the conventional immunosuppressive agents should be highly evaluated. Although more qualified evidence of beneficial effect of this therapy remains to be established in randomized controlled study, considering not only the high survival rate but the low cost for treatment of the complicated infections, the IVIg should be the potent inducible therapy which can be promoted before the beginning of conventional immunosuppressant treatment for relatively aged and lower immuno-potent MPO-ANCA patients in Japan.

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REFERENCES

1. Jennette, J. C., Falk, R. J., Andrassy, K., Bacon, P. A., Churg, J., Gross, W. L. et al. (1994): Nomenclature of systemic vasculitides. Proposal of an international consensus conference. Arthritis Rheum., 37, 187-192.
2. Jayne, D. R., Davies, M. J., Fox, C. J., Black, C. M. and Lockwood, C. M. (1991): Treatment of systemic vasculitis with pooled intravenous immunoglobulin. Lancet, 337, 1137-1139.
3. Richter, C., Schnabel, A., Csernok, E., De Groot, K., Reinhold-Keller, E. and Gross, W. L. (1995): Treatment of anti-neutrophil cytoplasmic antibody (ANCA)-associated systemic vasculitis with high-dose intravenous immunoglobulin. Clin. Exp. Immunol., 101, 2-7.
4. Jayne, D. R., Chapel, H., Adu, D., Misbah, S., O'Donoghue, D., Scott, D. et al. (2000): Intravenous immunoglobulin for ANCA-associated systemic vasculitis with persistent disease activity. Q. J. Med., 93, 433-439.
5. Sakai, H., Kurokawa, K., Koyama, A., Arimura, Y., Kida, H., Shigematsu, H. et al. (2002): Guidelines for the management of rapidly progressive glomerulonephritis. Jpn. J. Nephrol., 44, 55-82 (in Japanese).

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Editor-Communicated Paper

On the Cyto-Toxicity Caused by Quantum Dots

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Abstract: **Quantum dots (QDs) such as CdSe QDs have been introduced as new fluorophores. The QDs conjugated with antibody are starting to be widely used for immunostaining. However there is still not sufficient analysis of the toxicity of QDs in the literature. Therefore we evaluated the cell damage caused by the quantum dots for biological applications. We performed cell viability assay to determine the difference in cell damage depending on the sizes and colors of mercapto-undecanoic acid (MUA) QDs and the cell types. The results showed that the cell viability decreased with increasing concentration of MUA-QDs. But in the case of Vero cell (African green monkey's kidney cell) with red fluorescence QD (QD640), the cell damage was less than for the others. Furthermore through the flow cytometry assay we found that this cell damage caused by MUA-QD turned out to be cell death after 4–6-hr incubation. From the two assays described above, we found that there is a range of concentration of MUA-QDs where the cell viability decreased without cell death occurring and thus we conclude that attention should be given when MUA-QDs are applied to living organisms even in low concentrations.**

Key words: **Cell damage, MUA-QD, Cell death**

Quantum dots (QDs) such as CdSe QDs are nano-sized metal clusters. QDs have specific characteristics such as the quantum effect, which is a special photo quality caused by the widening of the band gap when the spatial dimension is reduced. Kubo et al. predicted the specific character of the quantum dot theoretically in 1962 (14–16). Since then, research concerning the applications of QDs has gained a great amount of interest. For example, in the field of Information Technology and optical-engineering (3, 10, 21, 29, 30), QDs have been proposed for use as a new material for memory, and as miniature laser-beam emitting devices. Furthermore, the biological applications of QDs conjugated with antibody have started to attract much attention, especially in immunostaining, separating cells, and diagnostics, because of their advantages such as longer lifetime and higher fluorescence over conventional organic fluorophores (1, 2, 8, 27). The first synthesized QDs are insoluble in biological solvents because non-polar groups of organic molecules are exposed on the surface of QDs. However the water-soluble QDs covered with mercapto-undecanoic acid (MUA) have been reported (2). In addition, the MUA-QD covered with sheep serum albumin (SSA) is well dispersed in water (2, 9). The advantages of MUA-QDs described above make it possible to consider the application of MUA-QDs to drug delivery systems (6, 20, 25, 28) as a drug-carrier and cell delivery system. Quantum dots have a longer lifetime compared to conventional organic fluorophores and thus make it easier to trace the drug delivered in living organisms. To make sure the application is feasible, an in-depth evaluation using MUA-QD in living organisms is needed. In fact cadmium (13) and selenium (24) are known to be toxic. Though the use of MUA-QDs for organisms has been known and some other studies about the actual injections into organisms have been conducted, the toxicity of MUA-QDs has not been reported in detail yet. Published works regarding

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Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; HC, human primary hepatocyte; MUA-QD, mercapto-undecanoic acid quantum dot; PBS, phosphate-buffered saline; PI, propidium iodide; SSA, sheep serum albumin; TOPO, tri-*n*-octylphosphine oxide.

QDs have so far only effectively assumed that QDs are safe. In this paper, we proceeded one step further by investigating the cell damage caused by MUA-QDs through an extensive and comprehensive experiment. We chose CdSe QDs because they are one of the QDs that have the strongest emission and they are used the most in many fields. In order to analyze the cell damage caused by MUA-QDs, a cell viability assay, which assesses the mechanism of glycolytic pathways, was conducted (12, 17, 26). Then in order to figure out whether the cell damage was cell death or not, we examined cell death using the flow cytometry assay.

Materials and Methods

Preparation of MUA-QDs. CdSe/ZnS QDs were synthesized in tri-*n*-octylphosphine oxide (TOPO) in accordance with the standard method (5, 11, 18, 19). For these experiments, three MUA-QDs were prepared; QD520, QD570 and QD640 which emitted green, yellow, and red, respectively.

Preparation of MUA-QDs solution with sheep serum albumin. The same volumes of 10 mg/ml MUA-QD and 10 mg/ml sheep serum albumin (SSA) were mixed as described in Hanaki et al. (9). Then we centrifuged this solution with a 0.45 μm filter at 5,000×*g* for 5 min at room temperature. The MUA-QD/SSA solution for all the cells was diluted with DMEM into several concentrations.

Cell viability assay. The cell viability was measured after the exposure of cells with MUA-QD to 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt to generate hormazan. The number of the living cells is known to be proportional to the concentration of the generated hormazan (12, 26). Cell viability was measured for the following two cell lines and a primary cell culture; Vero cells (African green monkey kidney cells), HeLa cells, and human primary hepatocyte (HC). The above-mentioned cell types were cultured at 37 C, in 5% CO_2 in DMEM, supplemented with 5% heat-inactivated fetal calf serum (FCS). All the cells were suspended in DMEM, supplemented with 5% FCS and 50 μg/ml gentamicin after they had been treated with trypsin and centrifuged at 1,800 rpm for 5 min at room temperature. The cell count was performed for the three types of cells respectively. Each cell was plated into a 96-well plate (Iwaki Co., Tokyo) at 3×10^4 cells/well (100 μl/well). After a 24-hr incubation, the DMEM was removed and the prepared MUA-QD/SSA solution diluted to several different concentrations was poured into the wells. After another 24-hr incubation period, a Cell Counting Kit8 (Dojindo Laboratories Co., Kumamoto, Japan) was added into the 110 μl/well. The Cell Counting Kit8 was diluted with DMEM (Cell Counting Kit8:DMEM=1:10). Then the absorbances were measured at 450 nm by an absorptiometer (Molecular Devices Co.).

Flow cytometry assay. For the flow cytometry assay (23), in all the experiments, each cell was plated into a 12-well plate (Iwaki Co., Tokyo) at 10^6 cells/well (1,000 μl/well).

The cells were incubated for 24 hr. The culture medium was removed, and then the prepared MUA-QD/SSA solution diluted to several different concentrations was poured into the wells. After incubation, the cells were washed with PBS and the dead cells were stained with propidium iodide (PI) (4, 7) (0.1 mg/ml) for 5 min at room temperature, followed by treatment with Puck's EDTA solution (4 mM, $NaHCO_3$; 136 mM, NaCl; 4 mM, KCl; 1 mM, EDTA; 1 mg/ml, glucose), which will do less damage to cells than trypsin. The cells were suspended in PBS after they were fixed with 3% formaldehyde. Then, the fluorescence intensity of PI and QD520 was measured using the flow cytometry (Cyto Ace 300 JASCO, Tokyo) assay.

Results and Discussion

Cell Viability Assay

We conducted the cell viability assay to confirm whether the MUA-QDs do damage to the cells or not (12, 26). We used three cell types; Vero cell, HeLa cell, and primary human hepatocyte for three MUA-QDs (QD520, QD570, and QD640). Their spectrums are shown in Fig. 1. The result showed that MUA-QDs affect the cell viability even in rather low concentrations (Fig. 2). The tendencies of the cell viability with QD570 and QD520 were almost the same. However only in the case of QD640 with Vero cells does the result show a difference in cell viability of less than 0.4 mg/ml. The cell damage was less than for the others only in this experiment though the tendency was the same.

Flow Cytometry Assay

Cell viability assay is easy to handle and quantitatively good as well. However, if the intracellular activity is affected; for example, that of NADH-Dehydrogenase, the results will not reflect the true number of cells. Therefore fluorescence intensity of PI was measured using flow cytometry; another method of counting living cells based on a different principle. Figure 3 shows the result of the experiment incubated for 24 hr with QD520 (23).

Collins et al. reported that living cells do not take in

propidium iodide (PI), which has 610 nm fluorescent (4). Only QD520 was used for the flow cytometry assay because the emission peaks of QD570 and QD640 could not be distinguished from that of PI. The top two Figs. (without MUA-QD) show that the emission intensity obtained with a PI filter and that obtained with a QD filter were both quite low. The emission intensity obtained from the PI filter, however, increased gradually, according to the concentration of the MUA-QDs. At more than 0.15 mg/ml concentration of MUA-QDs, the emission intensity of PI was split into two peaks; the left peak shows the living cells, and the right peak shows the dead cells. On the other hand, in the right lane (with the QD filter), in the cases where the concentration of MUA-QD was more than 0.15 mg/ml, the emission intensity of MUA-QD increased, depending on the concentration of MUA-QDs. The higher emission peak contains both the damaged cells and the undamaged cells in the left panel. At the same time, however, the intensity of PI also increased steadily, which means that the population of dead cells increased from 0.15 mg/ml upward. The results showed the cell damage caused by MUA-QD is cell death.

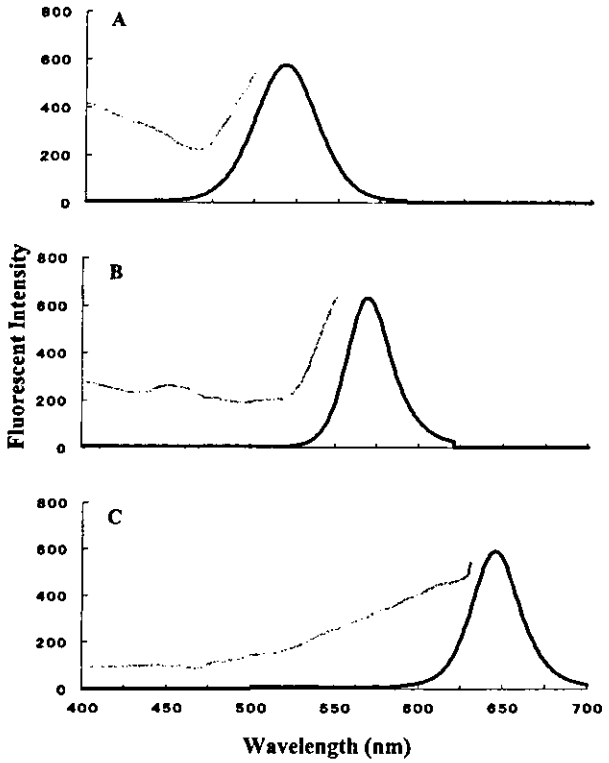

Fig. 1. Photoluminescent properties of three different MUA-QDs. (A) QD520, (B) QD570, and (C) QD640. Three different MUA-QDs were dissolved in DW, and their photoluminescent properties were measured with FP-6500. Emission spectra of QD520 excited at 300 nm, QD570 excited at 350 nm, and QD640 excited at 360 nm, represented as black lines. Excitation spectra represented as gray lines collected with detection at the respective peak spectra.

To analyze the dependence on the incubation time, we measured the ratio of the damaged cells (PI stained cells) against the total number of the cells chronologically (Fig. 4). The ratio of damaged cells increased sharply from 4-hr incubation in 0.2 mg/ml concentration of MUA-QD, and slowly in 0.1 mg/ml. On the other hand, we cannot observe any difference between the result obtained from the concentration of 0.05 mg/ml and that from the control. The result from the flow cytometry assay is compatible with that from the cell viability assay in the view of the concentration of MUA-QD causing cell damage. Cell damage caused by MUA-QD probably occurs because the connection of SSA that covers MUA-QD is not a chemical bond; it just attaches to the surface of the MUA-QD (9). Therefore SSA is easy to remove from the surface of MUA-QD and MUA comes out to the surface. To solve this problem, the surface-processing should be reexamined. Safer materials should be used to coat the surface of QDs or new safer QDs, such as silicon-QD, etc., can be considered for use for the DDS. As for its application for the DDS, the coating with peptide is effective because the tagging of target-molecules will be necessary: Peptide is more easily applicable for pharmaceutical biology and it is much safer. What is more, we have seen the difference in the extent of the cell damage only in the case of the combination of Vero cells and

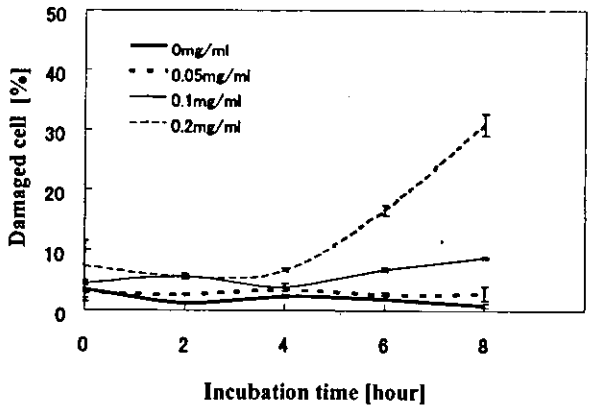

Fig. 4. Flow cytometry assay for the effect of the incubation time difference and concentration of QD. Vero cell and QD520 were used for this experiment. The vertical axis stands for the damaged cell % (the ratio of the number of the PI stained cell against the total number of cells). The intensity of PI is measured between 565 and 605 nm, and the intensity of QD520 is measured between 515 and 545 nm. The horizontal axis stands for the incubation time of the cell. The bold line stands for a concentration of QD520 at 0 mg/ml, the broken line stands for a concentration of 0.05 mg/ml, the solid line, 0.1 mg/ml, and the dotted line, 0.2 mg/ml. The vertical lines are the error bars.

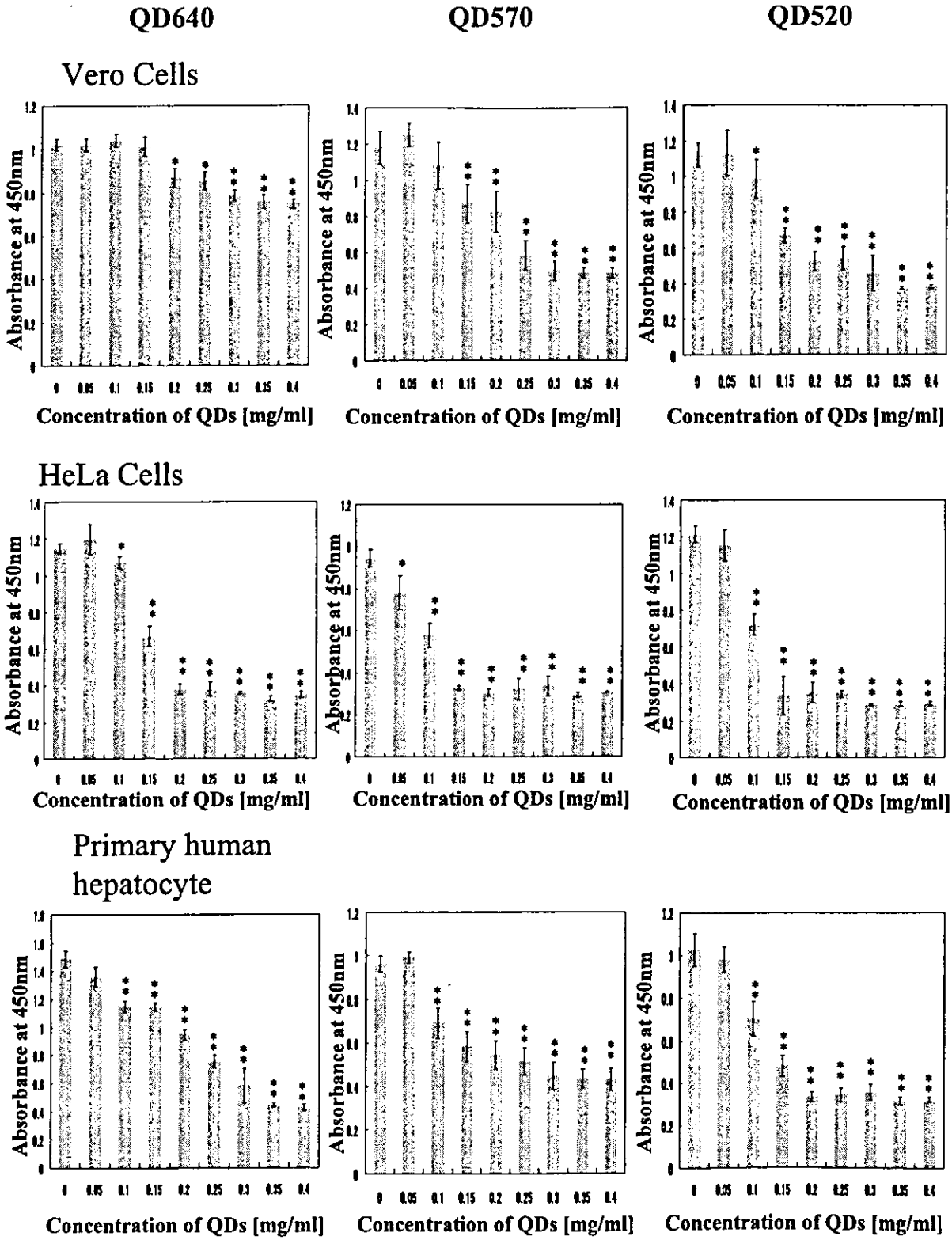

Fig. 2. Cell viability for the different sizes of the QDs and different cell types. Three of the different sizes of QDs are tested on the cell viability for each cell type (MTT assay, $n=5$). The top three panels stand for the cell viabilities of Vero cells, those in the middle for HeLa cells, and those in the bottom for primary human hepatocyte. The three panels in the left lane stand for QD640, those in the middle lane for QD570, and those in the right lane for QD520. In each panel, the horizontal axis stands for the concentration of QD, and the vertical axis stands for the absorbance at 450 nm. The columns in all the panels stand for the amount of hormazan, which reflect the cell viability, and I is standard deviation. A T-test was performed; * stands for the significance level <0.01, and ** stands for the significance level <0.001.

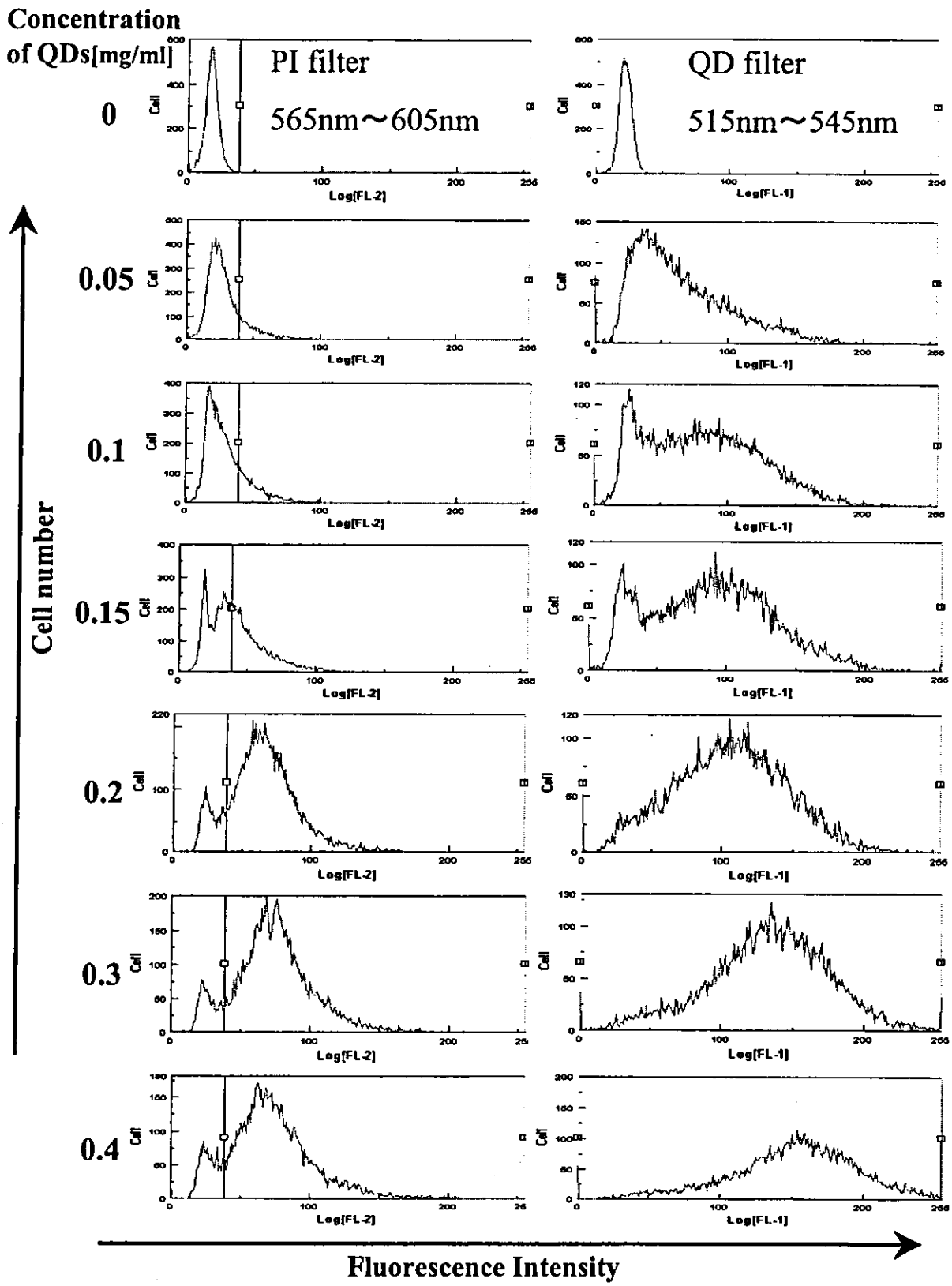

Fig. 3. Flow cytometry assay with the different concentrations of QD. Vero cell and QD520 are used for the flow cytometry assay. The horizontal axis, in the left lane, is the fluorescent intensity of propidium iodide with the filter (565 nm–605 nm), and in the right lane, the fluorescent intensity of QD520 with the filter (515 nm–545 nm). The vertical axes, in both the columns, stand for the cell count. Each row, from the top to the bottom, is given with respect to the concentration of QD520. In each row, the left panel and the right panel show the result with the same sample measured with a PI filter (left) and a QD filter (right), respectively.

QD640. It has been strongly suggested that the mobility of the MUA-QDs inside the cell depends on the size of the MUA-QDs (22). This might also explain the difference in the cell damage in our study.

In order to utilize quantum dots for humans, further study should be done on the relationship between the cell type and MUA-QD cell damage, an estimate of the mutation rate in bacteria and carcinogenesis in animals should be done and research into the mechanism of cyto-toxicity is needed. So far there is currently insufficient information about the discharge of MUA-QDs from living organisms.

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References

1) Bruchez, M., Jr., Moronne, M., Gin, P., Weiss, S., and Alivisatos, A.P. 1998. Semiconductor nanocrystals as fluorescent biological labels. Science **281**: 2013–2016.
2) Chan, W.C., and Nie, S. 1998. Quantum dot bioconjugates for ultrasensitive nonisotopic detection. Science **281**: 2016–2018.
3) Coe, S., Woo, W.K., Bawendi, M., and Bulovic, V. 2002. Electroluminescence from single monolayers in molecular organic devices. Nature **420**: 800–803.
4) Collins, A.M., and Donoghue, A.M. 1999. Viability assessment of honey bee, Apis mellifera, sperm using dual fluorescent staining. Theriogenology **51**: 1513–1523.
5) Dabbousi, B.O., Rodriguez-Viejo, J., Mikulec, F.V., Heine, J.R., Mattoussi, H., Ober, R., Jensen, K.F., and Bawendi, M.G. 1997. (CdSe) ZnS core-shell quantum dots: synthesis and characterization of a size series of highly luminescent nanocrystallites. J. Phys. Chem. B **101**: 9463–9475.
6) Dubertret, B., Skourides, D., Norris, J., Noireaux, V., Brivanlou, A.H., and Libchaber, A. 2002. *In vivo* imaging of quantum dots encapsulated in phospholipid micelles. Science **298**: 1759–1762.
7) Elstein, K.H., and Zucker, R.M. 1994. Comparison of cellular and nuclear flow cytometric techniques for discriminating apoptotic subpopulations. J. Neurochem. **74**: 1041–1048.
8) Gerion, D., Pinaud, F., Williams, S.C., Parak, W.J., Zanchet, D., Weiss, S., and Alivisatos, A.P. 2001. Synthesis and properties of biocompatible water-soluble silica-coated CdSe/ZnS semiconductor quantum dots. J. Phys. Chem. B **105**: 8861–8871.
9) Hanaki, K., Momo, A., Oku, T., Komoto, A., Maenosono, S., Yamaguchi, Y., and Yamamoto, K. 2003. Semiconductor quantum dot/albumin complex is a long-life and highly photostable endosome marker. Biochem. Biophys. Res. Commun. **302**: 496–501.
10) Harman, T.C., Taylor, P.J., Walsh, M.P., and LaForge, B.E. 2002. Quantum dot superlattice thermoelectric materials and devices. Science **297**: 2229–2232.
11) Hines, M.A., and Guyot-Sionnest, P. 1996. Synthesis and characterization of strongly luminescing ZnS-capped CdSe nanocrystals. J. Phys. Chem. **100**: 468–471.
12) Ishiyama, M., Miyazono, Y., Sasamoto, K., Ohkura, Y., and Ueno, K. 1997. A highly water-soluble disulfonated tetrazolium salt as achromogenic indicator for NADH as well as cell viability. Talanta **44**: 1299.
13) Kondoh, M., Araragi, S., Sato, K., Higashimoto, M., Takiguchi, M., and Sato, M. 2002. Cadmium induces apoptosis partly via caspase-9 activation in HL-60 cells. Toxicology **170**: 111–117.
14) Kubo, R. 1957. Statistical-mechanical theory of irreversible processes. I. J. Phys. Soc. Jpn. **12**: 570–586.
15) Kubo, R. 1962. Generalized cumulant expansion method. J. Phys. Soc. Jpn. **17**: 1100–1120.
16) Kubo, R. 1962. Stochastic liouville equations. J. Math. Phys. **4**: 174–183.
17) Mosman, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods **65**: 55–63.
18) Murray, C.B., Norris, D.J., and Bawendi, M.G. 1993. Synthesis and characterization of nearly monodisperse CdE (E=sulfur, selenium, tellurium) semiconductor nanocrystallities. J. Am. Chem. Soc. **155**: 8706–8715.
19) Peng, X., Schlamp, M.C., Kadavanich, A.V., and Alivisatos, A.P. 1997. Epitaxial growth of highly luminescent CdSe/CdS core/shell nanocrystals with photostability and electronic accessibility. J. Am. Chem. Soc. **119**: 7019–7029.
20) Rosenthal, S.J., Tomlison, I., Adkins, E.M., Schroeter, S., Adams, S., Swafford, L., McBride, J., Wang, Y., Defelice, L.J., and Blakely, R.D. 2002. Targeting cell surface receptors with ligand-conjugated nanocrystals. J. Am. Chem. Soc. **124**: 4586–4594.
21) Santori, C., Fattal, D., Vuckovic, J., Solomon, G.S., and Yamamoto, Y. 2002. Indistinguishable photons from a single-photon device. Nature **419**: 594–597.
22) Seydel, C. 2003. Quantum dots get wet. Science **300**: 80–81.
23) Shatrova, A.N., Aksenov, N.D., Poletaev, A.I., and Zenin, V.V. 2003. Effect of etoposide and amsacrine on mitotic progression of GM-130 and Hep-2 cell lines. The flow cytometry assay. Tsitologiia **45**: 59–68.
24) Shen, H.M., Yang, C.F., and Ong, C.N. 1999. Sodium selenite-induced oxidative stress and apoptosis in human hepatoma HepG2 cells. Int. J. Cancer **81**: 820–828.
25) Shubeita, G.T., Sekatskii, S.K., Dietler, G., Potapova, I., Mews, A., and Basch, T. 2003. Scanning near-field optical microscopy using semiconductor nanocrystals as a local fluorescence and fluorescence resonance energy transfer source. J. Microsc. **210**: 274–278.
26) Tominaga, H., Ishiyama, M., Ohseto, F., Sasamoto, K., Hamamoto, T., Suzuki, K., and Watanabe, M. 1999. A water-soluble tetrazolium salt useful for colorimetric cell viability assay. Anal. Commun. **36**: 47.
27) Wu, X., Liu, H., Liu, J., Haley, K.N., Treadway, J.A., Larson, J.P., Ge, N., Peale, F., and Bruchez, M.P. 2002. Immunofluorescent labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots.

Nat. Biotechnol. **21**: 41–46.

28) Xu, H., Sha, M.Y., Wong, E.Y., Uphoff, J., Xu, Y., Treadway, J.A., Truong, A., O'Brien, E., Asquith, S., Stubbins, M., Spurr, N.K., Lai, E.H., and Mahoney, W. 2003. Multiplexed SNP genotyping using the Qbead system: a quantum dot-encoded microsphere-based assay. Nucleic Acids Res. **31**: 43.

29) Wu, L.X., Steel, D.Y., Gammon, D., Stievater, T.H., Katzer, D.S., Park, D., Piermarocchi, C., and Sham, L.J. 2003. An all-optical quantum gate in a semiconductor quantum dot. Science **301**: 809–811.

30) Zrenner, A., Beham, E., Stufler, S., Findeis, F., Bichler, M., and Abstreiter, G. 2002. Coherent properties of a two-level system based on a quantum-dot photodiode. Nature **418**: 612–614.

Journal of Biomolecular NMR **29**: 543–544, 2004.

Letter to the Editor: ^{1}H, ^{13}C, ^{15}N resonance assignments of the cytokine LECT2

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Biological context

Human LECT2 (leukocyte cell-derived chemotaxin 2) is a 16-kDa chemotactic protein consisting of 133 amino acids and three intramolecular disulfide bonds. The protein was first purified from the culture fluid of phytohemagglutinin-activated human T-cell leukemia SKW-3 cells as a chemotactic factor to human neutrophils (Yamagoe et al., 1996) and its cDNAs were cloned from cDNA libraries of human, bovine, and murine livers (Yamagoe et al., 1998a,b). LECT2 is identical to chondromodulin-II (Hiraki et al., 1996), a bovine protein that stimulates the proliferation of chondrocytes and osteoblasts (Shukunami et al., 1999). A point mutation in LECT2 (Val58 to Ile58) is associated with the severity of rheumatoid arthritis (RA) (Kameoka et al., 2000). No tertiary structure has been solved so far for LECT2 and its related proteins. In order to reveal the three-dimensional structure of LECT2 and the effect of the point mutation on its conformation, we are doing NMR structural analysis of human LECT2. Here we report the ^{1}H, ^{15}N, and ^{13}C resonance assignments.

Methods and experiments

Human LECT2 (the 133-amino acid mature form, residue numbers 19–151) with an N-terminal His_6-tag was produced in *E. coli* as inclusion bodies, and renatured *in vitro* by a three-step refolding procedure (Ito et al., 2003). To prepare stable isotope-labeled protein, $^{15}NH_4Cl$ (> 99% ^{15}N) and ^{13}C-glucose (> 99% ^{13}C) were used as the sole nitrogen and carbon sources, respectively. The samples used for NMR measurements were 1 mM ^{15}N-labeled and $^{13}C/^{15}N$-labeled $(His)_6$-LECT2 dissolved in 50 mM Na_2SO_4 in 85% H_2O/10% D_2O/5% glycerol (pH 6.0, direct meter reading). NMR spectra were recorded at 298 K on Varian Unity Inova NMR spectrometers operated at ^{1}H frequencies of 500- and 750-MHz and equipped with triple-resonance z-gradient probes.

Sequence-specific backbone assignments were elucidated from 3D data of HN(CO)CA, HNCA, CBCA(CO)NH, HNCACB, CBCANH, HNCO, and (HCA)CO(CA)NH. C(CO)NH was used to confirm amino-acid types. For side-chain ^{1}H assignments, H(CCO)NH, HCCH-TOCSY, HCCH-COSY, ^{15}N-edited TOCSY, ^{15}N-edited NOESY, and 2D NOESY were used. ^{1}H chemical shifts were directly referenced to the resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), while ^{13}C and ^{15}N chemical shifts were referenced indirectly to DSS (Wishart et al., 1995). NMR data were processed using NMRPipe/NMRDraw (Delaglio et al., 1996). Visualization of transformed data and peak-picking were carried out using Sparky (http://www.cgl.ucsf.edu/home/sparky/). Secondary structure was predicted using CSI (Wishart and Sykes, 1994).

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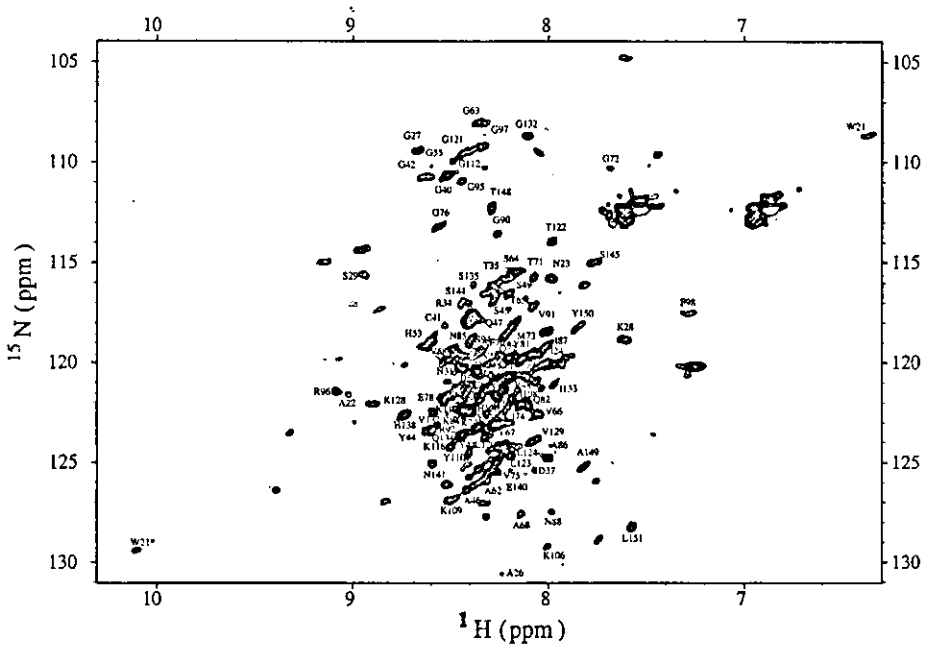

Figure 1. ^{1}H-^{15}N HSQC spectrum of $(His)_6$-LECT2 at 298 K. Amino acid labels were omitted from the middle of the HSQC for clarity. *indicates Trp side chain.

Extent of assignments and data deposition

Most of backbone resonances (90% of ^{15}N, 90% of H^N, 92% of C^α, 76% of H^α, 92% of C^β, and 79% of C′) and a part of aliphatic side-chain resonances have been assigned and deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under an accession number of 6025.

Figure 1 shows the ^{1}H-^{15}N HSQC spectrum of ^{15}N/^{13}C-labeled $(His)_6$-LECT2. A six-residue segment ranging from 100 to 105 and six other residues at positions 19, 24, 25, 38, 51 and 143 remain unassigned as well as the N-terminal His_6-tag. The assignments of these residues have been hampered due to severe overlaps of NMR signals and possible fast exchanges of H^N involved.

The secondary structure prediction by CSI indicates that LECT2 contains several β-strands but no α-helix, which is consistent with the far-UV CD (circular dichroism) data (Ito et al., 2003).

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References

Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J. and Bax, A. (1995) *J. Biomol. NMR*, **6**, 277–293.

Hiraki, Y., Inoue, H., Kondo, J., Kamizono, A., Yoshitake, Y., Shukunami, C. and Suzuki, F. (1996) *J. Biol. Chem.*, **271**, 22657–22662.

Ito, M., Nagata, K., Kato, Y. Oda, Y., Yamagoe, S., Suzuki, K. and Tanokura, M. (2003) *Protein Expr. Purif.*, **27**, 272–278.

Kameoka, Y., Yamagoe, S., Hatano, Y., Kasama, T. and Suzuki, K. (2000) *Arthritis Rheumatism*, **43**, 1419–1420.

Shukunami, C., Kondo, J., Wakai, H., Takahashi, K., Inoue, H., Kamizono, A. and Hiraki, Y. (1999) *J. Biochem.*, **125**, 436–442.

Wishart, D.S. and Sykes, B.D. (1994) *J. Biomol. NMR*, **4**, 171–180.

Wishart, D.S., Bigam, C.G., Yao, J., Abildaard, F., Dyson, H.J., Oldfield, E., Markley, J.L. and Sykes, B.D. (1995) *J. Biomol. NMR*, **6**, 135–140.

Yamagoe, S., Mizuno, S. and Suzuki, K. (1998a) *Biochim. Biophys. Acta*, **1396**, 105–113.

Yamagoe, S., Watanabe, T., Mizuno, S. and Suzuki, K. (1998b) *Gene*, **216**, 171–178.

Yamagoe, S., Yamakawa, Y., Matsuo, Y., Minowada, J., Mizuno, S. and Suzuki, K. (1996) *Immunol. Lett.*, **52**, 9–13.

Identification of the Leukocyte Cell-Derived Chemotaxin 2 as a Direct Target Gene of β-Catenin in the Liver

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To clarify molecular mechanisms underlying liver carcinogenesis induced by aberrant activation of Wnt pathway, we isolated the target genes of β-catenin from mice exhibiting constitutive activated β-catenin in the liver. Adenovirus-mediated expression of oncogenic β-catenin was used to isolate early targets of β-catenin in the liver. Suppression subtractive hybridization was used to identify the leukocyte cell-derived chemotaxin 2 (LECT2) gene as a direct target of β-catenin. Northern blot and immunohistochemical analyses demonstrated that LECT2 expression is specifically induced in different mouse models that express activated β-catenin in the liver. LECT2 expression was not activated in livers in which hepatocyte proliferation was induced by a β-catenin–independent signal. We characterized by mutagenesis the LEF/TCF site, which is crucial for LECT2 activation by β-catenin. We further characterized the chemotactic property of LECT2 for human neutrophils. Finally, we have shown an up-regulation of LECT2 in human liver tumors that expressed aberrant activation of β-catenin signaling; these tumors constituted a subset of hepatocellular carcinomas (HCC) and most of the hepatoblastomas that were studied. In conclusion, our results show that LECT2, which encodes a protein with chemotactic properties for human neutrophils, is a direct target gene of Wnt/β-catenin signaling in the liver. Since HCC develops mainly in patients with chronic hepatitis or cirrhosis induced by viral or inflammatory factors, understanding the role of LECT2 in liver carcinogenesis is of interest and may lead to new therapeutic perspectives. (HEPATOLOGY 2004;40:167–176.)

Hepatocellular carcinoma (HCC), the major primary liver cancer, is becoming increasingly common worldwide.[1] The prognosis for patients with HCC is rather poor. The molecular changes underlying HCC remain largely unknown despite the fact that major risk factors, such as chronic hepatitis B or C infection and exposure to hepatocarcinogens like aflatoxin B1, are well recognized. Several genetic changes have been implicated in at least 3 pathways of carcinogenesis, specifically, the p53, RB and Wnt/β-catenin signaling pathways.[2] Deregulation of the Wnt pathway appears to be most frequent of these changes in human HCC; it occurs in about 30% to 40% of patients.[2,3] It also occurs in more than 90% of hepatoblastomas, which are rare embryonal liver tumors.[4] Mutations affecting 2 partners of the Wnt pathway have been found in liver cancers. One is a mutation that activates the β-catenin gene. Such mutations occur mainly in hepatitis B-negative HCC[5] and in more than 50% of hepatoblastomas.[6,7] The other is a mutation that inactivates the axin 1, and, less commonly, the axin 2 gene.[5,8,9] Mutations that activate the Wnt pathway result in β-catenin accumulation in the nucleus. This process, in association with LEF/TCF transcription factors, modulates the transcription of target genes.[10,11] It is now clear that the genetic program triggered by activation of β-catenin signaling depends on the cellular context. The β-catenin target genes c-myc and cyclin D1 are well

Abbreviations: HCC, hepatocellular carcinoma; GS, glutamine synthetase; LECT2, leukocyte cell-derived chemotaxin 2; SSH, suppression subtractive hybridization; cDNA, complementary DNA; PMN, polymorphonuclear leukocytes; PCR, polymerase chain reaction; HBSS, Hanks' Balanced Salt Solution; ISH, in situ *hybridization; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; fMLP, N-formyl-methionyl-leucyl-phenylalanine; RT-PCR, reverse-transcriptase polymerase chain reaction; HA-tagged, influenza hemagglutinin-tagged.*

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recognized[12,13]; neither c-myc nor cyclin D1 were induced in the liver of transgenic mice that express an oncogenic form of β-catenin, although such mice exhibit hepatomegaly and marked hepatocellular proliferation.[14] We used several mouse models in which β-catenin signaling in the liver is activated to identify liver-specific target genes of the Wnt pathway that may be implicated in the development of liver cancer. We have identified 3 components of the metabolic pathway of glutamine and have demonstrated that the glutamine synthetase (GS) gene, which is frequently overexpressed in HCC, is a target of β-catenin signaling.[15]

To search for early genes that are sensitive to deregulation of the Wnt/β-catenin pathway in the liver, we used mice infected with an adenovirus that encodes an oncogenic form of β-catenin. We have previously shown that activation of the Wnt pathway using this approach may be achieved as early as 48 hours postinfection.[15] This report describes the identification of a new β-catenin target gene, leukocyte cell-derived chemotaxin 2 (LECT2), which is expressed in the liver.

Materials and Methods

Animals. All procedures involving animals reported in this paper were carried out in accordance with French government regulations (Services Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture). L-PK/c-myc,[3] ΔN131β-catenin,[14] and ASV[16] transgenic mice have been previously described.

Adenoviral Gene Transfer. The adenoviruses AdGFP, AdLacZ, and AdβcatS37A have been described previously.[17] B6D2/F1 mice were injected intravenously with 5×10^9 plaque-forming units of AdGFP, AdLacZ, or AdβcatS37A, and were sacrificed 48 hours later.

Suppression Subtractive Hybridization (SSH). We generated complementary DNA (cDNA) from 1 μg poly(A)+ RNA isolated from the livers of 3 mice injected intravenously with AdβcatS37A (tester) and 6 mice injected intravenously with AdLacZ or AdGFP (driver) using the SMART PCR cDNA Synthesis Kit (BD Biosciences, Paris, France). SSH was undertaken using the PCR-Select cDNA Subtraction Kit (BD Biosciences, Clontech, Palo Alto, CA) according to the manufacturer's protocol. The subtracted cDNA library was subcloned into T/A cloning vector pT-Adv (Clontech, Paris, France) and transformed to ElectroMAX DH10B cells (Invitrogen, Carlsbad, CA). The library was plated on LB-ampicillin plates and incubated at 37°C overnight. Individual clones of the library were plated on LB-ampicillin 96 plates and 2 replicas were made using Hybond N+ (Amersham Biosciences, UK) nylon membranes. For differential screening, replica filters were hybridized with ^{32}P-labeled subtracted tester end driver probes. Blast search was used to analyze sequence homologies in the gene database.

Human Tumor Samples and RNA Sources. All tumor samples were obtained from surgical liver resections. RNA samples were extracted from frozen liver sections. HCC RNA samples were obtained; 29 RNA samples were kindly provided by Dr. Marie-Annick Buendia (Institut Pasteur, France) and 22 RNA samples were extracted from tumor samples obtained at Cochin Hospital (Paris, France). These tumor samples were evaluated for the presence of mutations that activate the β-catenin gene as previously described.[3] Liver specimens were obtained from patients with hepatoblastoma managed at the Bicêtre Hospital (Le Kremlin-Bicêtre, France). All of these patients had received preoperative chemotherapy. Samples were fixed in 10% neutral buffered formalin and were embedded in paraffin. Hepatoblastoma RNA samples were kindly provided by Dr Marie-Annick Buendia (Institut Pasteur, France). According to French law and ethical guidelines, no informed consent is required before analysis of RNA samples from specimens of resected tissue that would otherwise be discarded.

Preparation of Polymorphonuclear Leukocytes. Heparinized human venous blood was obtained from healthy volunteers. Polymorphonuclear leukocytes (PMN) were isolated using a 2-step sedimentation. Whole heparinized (10 units/mL) blood on 2% Dextran T500 in saline was centrifuged, and the granulocyte-rich supernatant was then centrifuged on a Ficoll-Hypaque cushion (Eurobio, Paris, France), as previously described.[18] Purified PMN (95%-97 %) were subjected to hypotonic lysis for 30 seconds, washed, and suspended in Hanks' Balanced Salt Solution (HBSS) containing 1.2 mmol calcium at pH 7.4.

Cell Transfection Studies. Huh7 and HepG2 cells were maintained in DMEM containing 10% (vol/vol) calf serum. Transient transfections were undertaken when cells were 60% to 70% confluent in 12-well plates, using Lipofectamine Plus Reagent (Invitrogen). A TK-Renilla plasmid (10 ng) was included in each transfection as a reference for monitoring transfection efficiency. Cells were lysed 24 hours after transfection and the luciferase and Renilla activities were assayed using Dual Luciferase Reporter Assay (Promega, Madison, WI). All experiments were undertaken in duplicate and were repeated at least 3 times. The total amount of transfected DNA was kept constant by adding the empty expresssion vector pCAN. The ΔN89β-catenin-pCAN expression vector was kindly provided by P. Polakis (San Francisco, CA), and the expression plasmid encoding