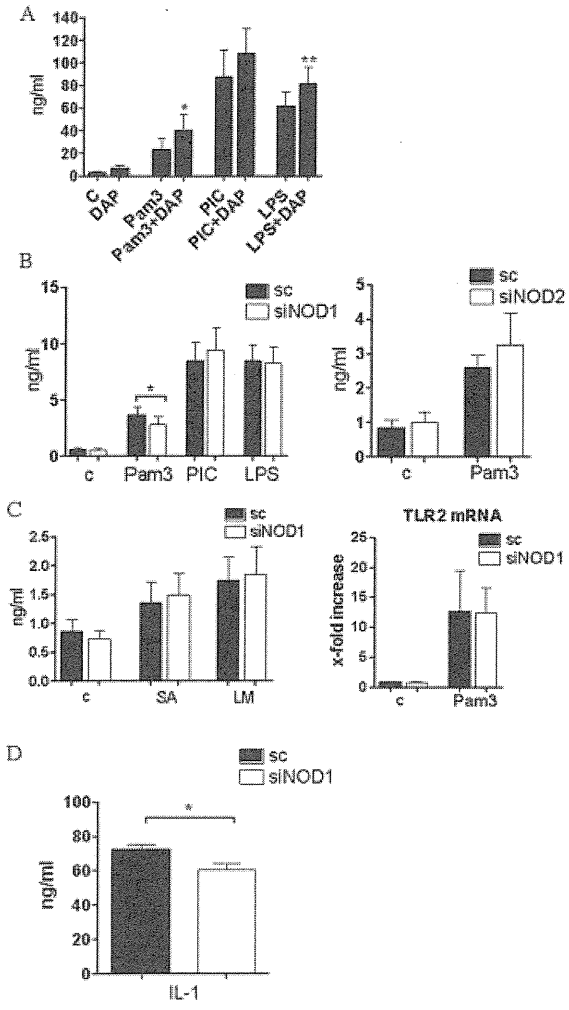

Figure 5. Down-regulation of Toll-like receptor 2 (TLR-2) and interleukin-1β (IL-1β)–induced production of IL-6 by silencing of NOD-1 in RA synovial fibroblasts (RASFs). **A,** Two-way analysis of variance showed synergistic interaction of L-alanyl-γ-D-glutamyl-meso-diaminopimelic acid (Tri-DAP [DAP]) with palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK_4 [Pam3]) and lipopolysaccharide (LPS), but not with poly(I-C) (PIC), in the induction of IL-6 production in RASFs (n = 6). **B,** Knockdown of NOD-1 with small interfering RNA (siNOD1) in RASFs (n = 6) led to significantly decreased levels of IL-6, when compared to those in scrambled control siRNA (sc)–transfected RASFs, after Pam_3CSK_4 stimulation (left). Knockdown of NOD-2 did not induce any change in the IL-6 levels in RASFs (n = 4) after stimulation with Pam_3CSK_4 (right). **C,** Stimulation with heat-inactivated *Staphylococcus aureus* (SA) or *Listeria monocytogenes* (LM) resulted in similar levels of IL-6 in NOD-1 siRNA–transfected cells and control siRNA–transfected cells (each n = 6) (left). After stimulation with Pam_3CSK_4, TLR-2 mRNA levels were increased in both NOD-1 siRNA– and control siRNA–transfected RASFs (each n = 3) (right). **D,** IL-6 levels were significantly lower in siNOD-1–transfected cells compared with control siRNA–transfected cells (each n = 6) after stimulation with IL-1β. * = $P < 0.05$; ** = $P < 0.01$ versus control, by Wilcoxon's matched pairs test. In **A–C,** unstimulated cells were used as treatment controls (C). Bars show the mean ± SEM. See Figure 1 for other definitions.

role in TLR-2 signaling (Figure 5B, left). No such effect was seen when NOD-2 was knocked down, as shown by the similar levels of IL-6 after TLR-2 stimulation in control siRNA–transfected cells and siNOD-2–transfected cells (Figure 5B, right).

We then tested whether the modulating effect of NOD-1 would still occur if the TLR-2 pathways were not selectively activated, and whether it must act in combination with other PRRs. The cell wall of *Staphylococcus aureus* contains TLR-2–activating peptidoglycans as well as NOD-2–activating muramyl dipeptides (MDPs). The cell wall of *Listeria monocytogenes* contains, in addition to these molecules, DAP, and therefore stimulates the TLR-2, NOD-2, and NOD-1 pathways. Our experiments with these bacterial agents showed that the double and triple activation of PRRs overruled the modulating effect of NOD-1 seen with specific TLR-2 stimulation alone, and no difference in the production of IL-6 was observed between NOD-1–silenced and control siRNA–transfected RASFs (Figure 5C, left).

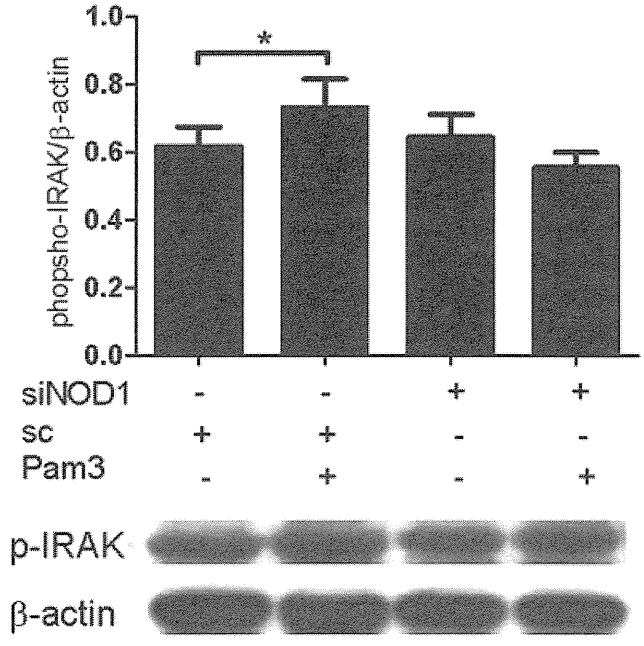

Figure 6. Influence of NOD-1 on interleukin-1 receptor–associated kinase 1 (IRAK-1) phosphorylation in RA synovial fibroblasts (RASFs). After stimulation of RASFs with palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK_4 [Pam3]) for 20 minutes, the phosphorylation of IRAK-1, expressed as the ratio of phospho–IRAK-1 to β-actin, significantly increased in scrambled control (sc) small interfering RNA (siRNA)–transfected RASFs, but not in NOD-1 siRNA–transfected cells (top). Western blots of IRAK-1 phosphorylation in RASFs from each group are also shown, with β-actin used as a positive control (bottom). Bars show the mean ± SEM of 15 samples per group. * = $P < 0.01$ by Wilcoxon's matched pairs test. See Figure 1 for other definitions.

Since this specific effect of NOD-1 silencing on TLR-2 signaling could be due to the down-regulation of TLR-2 itself as a result of the knockdown of NOD-1, we measured TLR-2 transcripts after the silencing of NOD-1. As described above, the levels of TLR-2 were strongly increased after incubation of the RASFs with the TLR-2 ligand. Silencing of NOD-1 had no effect on the TLR-2 levels in either unstimulated or stimulated cells (Figure 5C, right).

Among the 3 TLRs analyzed, TLR-2 is the only one that exclusively signals via the adaptor protein myeloid differentiation factor 88 (MyD88). Therefore, we hypothesized that NOD-1 might influence the MyD88 signaling pathway. If this were the case, then NOD-1 knockdown should diminish IL-1 signaling, since MydD88 is also recruited by the IL-1 receptor after ligand binding. Stimulation of NOD-1–knockdown cells with IL-1β led to an 18% reduction in the levels of IL-6 when compared to that in IL-1β–stimulated control siRNA–transfected cells (Figure 5D), corroborating a modulating role of NOD-1 in the MyD88 pathway.

Activation of MyD88 leads to phosphorylation of IRAK-1. Accordingly, we found that stimulation of RASFs with Pam_3CSK_4 for 20 minutes increased the phosphorylation of IRAK-1 (Figure 6). Knockdown of NOD-1, however, prevented the phosphorylation of IRAK-1 after stimulation with Pam_3CSK_4.

DISCUSSION

In the present study, we have shown that NOD-1 is strongly expressed in RA synovium, and that its expression can be induced in RASFs by stimulation of TLR-3. NOD-1 stimulation of RASFs led to a rapid increase in the production of proinflammatory and matrix-degrading mediators, followed by up-regulation of the expression of TLR-2 and NOD-2, and this activity of NOD-1 synergized with the stimulatory effects of TLR-2 and TLR-4 in the production of IL-6. Furthermore, knockdown of NOD-1 diminished the production of IL-6 after stimulation with Pam_3CSK_4 and IL-1β, and blocked the phosphorylation of IRAK-1.

As our results show, NOD-1 expression was increased equally in synovial fibroblasts, macrophages, and PBMCs. Furthermore, TLR-3 stimulation further increased the expression of NOD-1 in RASFs. The high levels of NOD-1 in RA synovial tissue, compared to OA synovial tissue, can therefore be attributed, most probably, to the influx of immune cells in the synovium, in conjunction with the higher NOD-1 expression in RASFs caused by the activation of TLR-3. It has been shown that endogenous double-stranded RNA from necrotic cells can activate RASFs via TLR-3, which might be the mechanism by which expression of NOD-1 is increased in RASFs in vivo (5). The high expression of NOD-1 observed in the synovial tissue of patients with gout indicates that NOD-1 may play a pathophysiologic role in this disease as well. Similar to the NLRP3 inflammasome, which is well known to play an important role in gout, NOD-1 has been shown to bind to caspase 1 and promote IL-1 secretion (19).

Stimulation of NOD-1 led to the production of a wide range of proinflammatory mediators and MMPs in RASFs. In addition, there was a synergistic effect on the production of IL-6 in RASFs following simultaneous stimulation of NOD-1 and the TLRs. Surprisingly, HMDMs reacted to a much lower extent to stimulation with Tri-DAP than did synovial fibroblasts. Taken together, these findings highlight the important role of synovial fibroblasts as cells of the innate immune system that rapidly integrate and elicit an innate immune response. Of note, stimulation of NOD-1 selectively induced the increased expression of other peptidoglycan-sensing PRRs, namely, TLR-2 and NOD-2, but not TLR-3 and TLR-4. This indicates that there may be a directed chain reaction in the mechanisms of proper immune defense, rather than a general increase in PRRs after sensing of invading pathogens.

Exogenous ligands for PRRs, such as the NOD-2 ligand MDP, or bacterial peptidoglycans have been identified in the joints of patients with RA (18,20). Moreover, the list of endogenous ligands for PRRs continues to expand, and it has become clear that, in addition to their role in immune defense, PRRs are important sensors of tissue damage. No endogenous ligands for the NLRs NOD-1 and NOD-2 have been found up to now. In addition, it should be noted that direct interactions of NOD-1 and NOD-2 with their respective ligands have also not as yet been demonstrated. Therefore, the possibility that these NLRs respond to their ligands via an indirect mechanism cannot be excluded.

The results presented herein suggest that the presence of NOD-1 is essential for the phosphorylation of IRAK-1 in RASFs after recruitment in response to MyD88. This is particularly interesting since NLRP12, a member of the pyrin domain–containing NLR subfamily, has been shown to bind IRAK-1 and to block its phosphorylation (21). In general, accumulating evidence indicates that NLRs tend to associate with other proteins to form large complexes, and that the composition

of these complexes will determine the biologic function of the various NLRs (22).

In summary, the results of the present study demonstrate that the expression of NOD-1 is increased in the synovial tissue of RA patients, and that RASFs show a strong proinflammatory response after stimulation of NOD-1 with its ligand, Tri-DAP. Moreover, down-regulation of NOD-1 leads to reduced levels of IL-6 in RASFs after stimulation with TLR-2 or IL-1β, and will result in blocked phosphorylation of IRAK-1. Thus, our findings indicate that NOD-1, either alone or in interactions with other inflammatory mediators, plays an important role in the chronic and destructive joint inflammation in RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Ospelt had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Yokota, R. E. Gay, S. Gay, Ospelt.

Acquisition of data. Yokota, Miyazaki, Kolling, Fearon, Suzuki, Ospelt.

Analysis and interpretation of data. Yokota, Hemmatazad, R. E. Gay, Fearon, Mimura, S. Gay, Ospelt.

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ORIGINAL ARTICLE

Serum osteoprotegerin concentration is associated with carotid atherosclerotic plaque in patients with rheumatoid arthritis

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Abstract
Objective Osteoprotegerin (OPG), a regulator of bone resorption, is involved in the pathogenesis of rheumatoid arthritis (RA) and atherosclerosis. OPG is elevated in patients with coronary artery disease, and high OPG levels are associated with cardiac disease severity and mortality in the general population. The purpose of this study was to investigate the relationship of serum OPG levels, traditional coronary risk factors, and RA-related factors to carotid atherosclerosis in RA patients.
Methods Ninety-one RA patients were studied (85 % women, age 60 ± 10 years). Serum OPG levels were measured by an enzyme-linked immunosorbent assay. The prevalence of carotid plaque was assessed by ultrasonographic imaging in all patients. The relationship between various clinical characteristics, OPG, and carotid plaque was examined.
Results Serum OPG levels were significantly higher in patients with carotid plaque than in those without plaque (median level 1,397 vs. 887 pg/mL, respectively; $P = 0.006$). There were no significant differences between RA patients with and without carotid plaque with respect to sex, duration of RA, blood pressure, body mass index, smoking, low-density lipoprotein cholesterol, Disease Activity Score-28, van der Heijde-modified Sharp score, and prednisolone dose. After adjusting for age, sex, and C-reactive protein, elevated levels of OPG were still associated with a higher prevalence of carotid plaque in patients with RA ($P = 0.038$).
Conclusion RA patients suffer from accelerated atherosclerosis and also have increased levels of OPG. The serum OPG level is independently associated with carotid plaque.

Keywords Osteoprotegerin · Rheumatoid arthritis · Atherosclerosis

Introduction

Previous clinical studies have demonstrated that patients with rheumatoid arthritis (RA) show a marked increase of atherosclerosis and a higher prevalence of cardiovascular disease compared with the age-matched general population. The excess mortality and morbidity of RA patients is thought to be due to an increased incidence of myocardial infarction [1]. Two markers of premature atherosclerosis, carotid artery plaque and coronary artery calcification, show an increased prevalence in patients with RA [2, 3], but the excess co-morbidity from accelerated atherosclerosis in RA patients is not fully explained by established coronary risk factors [4]. Mechanisms involved in the pathogenesis of atherosclerosis and RA are thought to be shared. In the general population, it is becoming increasingly clear that inflammation plays an important role in atherogenesis. Thus, chronic inflammation and other factors may enhance the development of atherosclerosis in RA patients.

Mortality from coronary heart disease (CHD) is much lower in Japan than in the USA or Europe according to an

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analysis of death certificates from the World Health Organization database. In Japan, CHD mortality is 36 per 100,000 for men and 18 per 100,000 for women [5]. In comparison, in the USA, CHD mortality is 121 per 100,000 for men and 67 per 100,000 for women, and in the European Union, it is 100 per 100,000 for men and 45 per 100,000 for women. The incidence rate of acute myocardial infarction is also much lower in Japan than in the USA (63 vs. 230 per 100,000, respectively) [6, 7]. This lower incidence rate of atherosclerotic cardiovascular disease in the Japanese population may be due to the weaker influence of traditional coronary risk factors. On the other hand, Japanese RA patients (like Caucasian patients) have a larger carotid artery intima-media thickness than healthy controls [8, 9]. Therefore, it seems important to identify risk factors for accelerated atherosclerosis other than the traditional factors in Japanese patients with RA.

Osteoprotegerin (OPG) is a decoy receptor activator for nuclear factor κB ligand (RANKL) that acts as a regulator of bone resorption, immunity, and cardiovascular function. OPG expression has been detected in human atherosclerotic plaque as well as in osteoblasts [10, 11]. The role of OPG in atherogenesis has recently been elucidated. It is secreted by endothelial cells and smooth muscle cells in response to stimulation by proinflammatory cytokines, and it up-regulates the expression of endothelial adhesion molecules that facilitate the migration of monocytes and lymphocytes into the vascular intima during the process of atherogenesis. OPG is also related to plaque rupture [12, 13]. In addition, serum OPG concentrations are elevated in patients with coronary artery disease, and the OPG level is associated with cardiovascular disease severity and increased cardiovascular mortality in the general population [14–16]. OPG is also expressed in synovial tissue obtained from the joints of RA patients [17], suggesting a possible role in the pathogenesis of both atherosclerosis and RA.

In the study reported here, we explored the hypothesis that Japanese patients with RA have increased OPG levels and whether these are associated with accelerated atherosclerosis.

Patients and methods

Subjects

We studied 91 patients with RA (84.6 % women, aged 60 ± 10 years). All of the patients were over 20 years old and fulfilled the criteria of the American College of Rheumatology for a diagnosis of RA. They were recruited during regular visits with their rheumatologists at the Saitama Medical University Hospital [18]. Patients with a history of cardiovascular disease (previous stroke, myocardial infarction or angina) were excluded. This study was approved by the Institutional Review Board of Saitama Medical University Hospital, and all subjects gave written informed consent.

Clinical assessment

Data on demographic characteristics and clinical characteristics were collected. Clinical information was obtained through a structured interview and physical examination, as well as by a review of the medical records. Various risk factors for atherosclerosis were investigated, including age, hypertension, cigarette smoking, diabetes, obesity, hypercholesterolemia, and a family history of cardiovascular disease (including myocardial infarction or stroke). Subjects were considered to have hypertension if they were taking antihypertensive drugs or if they had a systolic blood pressure ≥140 mmHg and/or a diastolic blood pressure ≥90 mmHg. Obesity was assessed from the body mass index. RA disease activity was assessed by using the Disease Activity Score 28 (DAS28) based on evaluation of 28 joints, the erythrocyte sedimentation rate (ESR), and global assessment of the patient's well-being [19]. Radiographs of the hands and feet were reviewed for all patients. These radiographs were obtained within 3 months before or after the date of collecting blood samples. Radiographic joint damage was assessed according to the van der Heijde-modified Sharp score [20], with the number and size of bone erosions and the extent of joint space narrowing related to the cartilage damage being evaluated. Current medications, the daily dose of prednisolone (PSL), the weekly dose of methotrexate (MTX), and the use of biologics were determined by combining information provided by the patients and the medical records.

Measurement of serum OPG

Blood samples were collected from all patients after an overnight fast, and the serum was separated by centrifugation of the blood samples and stored at −70 °C until assayed. The serum OPG concentration was measured with a commercial enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (R&D systems, Minneapolis, MN). Monoclonal mouse anti-human OPG antibody was used as the capture antibody and biotinylated polyclonal goat anti-human OPG antibody was used for detection. The mean coefficient of variation was 4.3 %.

Other laboratory tests

Routine biochemistry tests, markers of inflammation, such as ESR and C-reactive protein (CRP), and markers of RA,

such as rheumatoid factor and matrix metalloproteinase-3 (MMP-3), were measured.

Carotid artery ultrasonography

Carotid artery ultrasonography was performed by experienced sonographers according to the same protocol for all patients using a high-resolution B-mode ultrasound device (Aplio MX; Toshiba, Medical Systems, Tochigi, Japan; or ProSound II SSD-6500; Aloka, Tokyo, Japan). Atherosclerotic plaque was defined as a lesion protruding into the vessel lumen at least 50 % beyond the diameter of the surrounding wall. Plaques were measured in the near and far walls of the right and left common carotid arteries, the bifurcation, and the internal carotid arteries.

Statistical analysis

Characteristics of patients are shown as the mean ± SD or as the median for continuous variables, while categorical variables are shown as frequencies and percentages. Clinical characteristics were compared between patients with and without carotid plaque by using the Mann–Whitney *U* test and Fisher's exact test. Logistic regression analysis was employed to assess the influence of age and sex or age, sex, and CRP on the presence of carotid plaque. All analyses were performed using SPSS for Windows, ver. 18.0 (IBM Japan, Tokyo, Japan).

Results

Demographic profile

The 91 RA patients were predominantly female ($n = 77$, 84.6 %) and their age ranged from 22 to 78 years (mean age 60 ± 10 years; median age 60 years). The disease duration of the patients ranged from 0.2 to 58 years (mean duration 11 ± 9 years; median duration 10 years). Carotid plaque was detected by ultrasonography in 38 patients with RA (41.8 %), of whom 31 were female (40.3 % of female patients). The serum OPG concentration did not differ significantly ($P = 0.448$) between female and male patients (1,232 ± 780 vs.1,369 ± 911 pg/mL, respectively).

Prevalence of carotid plaque

The prevalence of carotid plaque was investigated both in all 91 patients with RA and in the 77 female patients with RA across different age groups. The prevalence of carotid plaque was found to increase with increasing age (Fig. 1). Prevalence rates of carotid plaque ranged from 0 % for patients under the age of 40 years to 64 % for those patients aged >70 years. The prevalence trends of carotid plaque in the group of female RA patients did not differ from those for all 91 patients with RA.

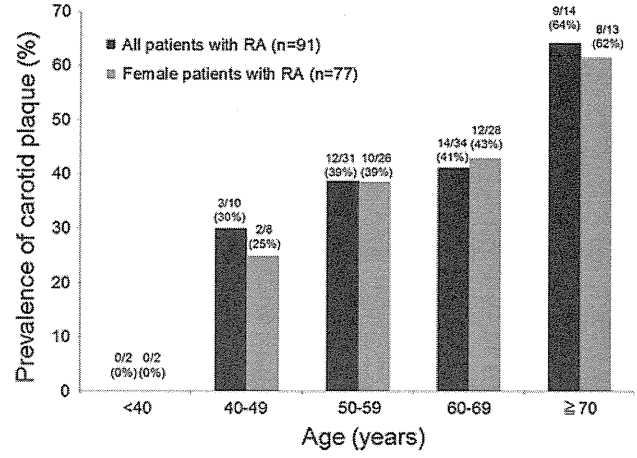

Fig. 1 Prevalence of carotid plaque by age strata in patients with rheumatoid arthritis (*RA*). The prevalence of carotid plaque increased with age in both the group consisting of all patients with RA ($n = 91$) and that of only female patients with RA ($n = 77$)

Comparison of coronary risk factors, disease activity, joint damage, and medications between patients with and without carotid plaque

The characteristics of the patients with or without carotid plaque are shown in Table 1. Compared to patients without plaque, those who had plaque were older (median age 64 vs. 59 years; $P = 0.009$). There was a similar sex ratio among patients with and without plaque (13 and 18 % male, respectively; $P = 0.35$). No significant differences were found with respect to established risk factors for atherosclerosis, such as blood pressure, BMI, low-density lipoprotein (LDL) cholesterol, and current smoking, but older age was a significant risk factor. Postmenopausal status (only in female patients) was more common in patients with carotid plaque than in those without plaque, but the differences were not significant. The duration of RA, DAS28 score, rheumatoid factor, severity of joint damage as defined by radiography, and current use of PSL, MTX, or biologics did not differ between patients with and without carotid plaque. MMP-3 levels were significantly higher in patients with carotid plaque than in those without plaque (median 191 vs. 123 ng/mL, respectively; $P = 0.03$), but this difference disappeared after adjustment for age and sex.

Association of OPG with carotid atherosclerotic plaque in patients with RA

Serum OPG concentrations were significantly higher in the patients with carotid plaque than in those without plaque

Table 1 Characteristics of patients with rheumatoid arthritis according to the presence or absence of carotid plaque

Variable	No plaque ($n = 53$)	Plaque ($n = 38$)	P value	Age and sex adjusted	
				Odds ratio (95 % CI)	P value
Age (years)	57 ± 11 (59)	63 ± 9 (64)	0.009	NA	NA
Male (%)	13	18	0.35[a]	NA	NA
Disease duration (years)	11 ± 7 (10)	12 ± 11 (12)	0.90	1.003 (0.955–1.054)	0.90
Current smoker (%)	11	14	0.76[a]	1.242 (0.274–5.618)	0.78
Family history of CHD (%)	25	23	1.00[a]	0.829 (0.289–2.385)	0.73
Post menopausal (% in female)	78	93	0.11[a]	1.496 (0.200–11.15)	0.69
Systolic blood pressure (mmHg)	124 ± 18 (122)	125 ± 15 (125)	0.49	0.996 (0.970–1.024)	0.79
Diastolic blood pressure (mmHg)	73 ± 11 (70)	71 ± 8 (70)	0.46	0.982 (0.938–1.029)	0.46
Hypertension (%)	42	29	0.27[a]	0.480 (0.189–1.219)	0.12
Body mass index (kg/m^2)	21.4 ± 3.0 (21.2)	21.0 ± 4.1 (21.5)	0.81	0.970 (0.854–1.102)	0.64
Hemoglobin (g/dL)	12.1 ± 1.9 (12.0)	12.2 ± 1.8 (11.9)	0.89	1.158 (0.893–1.502)	0.27
Albumin (g/dL)	4.1 ± 0.5 (4.1)	3.9 ± 0.5 (4.1)	0.12	0.791 (0.301–2.075)	0.63
Creatinine (mg/dL)	0.7 ± 0.8 (0.6)	0.7 ± 0.3 (0.6)	0.76	0.666 (0.270–1.640)	0.38
Total cholesterol (mg/dL)	193.2 ± 28.2 (195.0)	191.6 ± 32.6 (186.0)	0.61	0.998 (0.983–1.013)	0.79
HDL cholesterol (mg/dL)	59.1 ± 15.3 (57.5)	63.8 ± 19.3 (59.5)	0.43	1.015 (0.983–1.047)	0.36
LDL cholesterol (mg/dL)	112.8 ± 25.3 (116.0)	103.2 ± 28.6 (101.5)	0.09	0.986 (0.969–1.005)	0.15
Triglycerides (mg/dL)	99.9 ± 38.5 (89.0)	106.3 ± 53.5 (88.0)	0.97	1.006 (0.995–1.016)	0.28
Fasting blood glucose (mg/dL)	102 ± 22 (96)	102 ± 29 (93)	0.56	0.996 (0.977–1.016)	0.72
HbA1c (%)	5.9 ± 6.0 (5.1)	5.3 ± 0.6 (5.3)	0.06	0.975 (0.836–1.138)	0.75
Uric acid (mg/dL)	4.6 ± 1.2 (4.5)	4.6 ± 1.2 (4.5)	0.97	0.928 (0.641–1.342)	0.59
Tender joint counts (28 joints)	4 ± 5 (2)	4 ± 5 (2)	0.63	1.013 (0.926–1.110)	0.77
Swollen joint counts (28 joints)	5 ± 4 (3)	3 ± 4 (2)	0.12	0.923 (0.824–1.035)	0.17
ESR (mm/h)	47 ± 36 (36)	60 ± 41 (53)	0.17	1.002 (0.990–1.015)	0.71
DAS28 (ESR4)	4.3 ± 1.4 (4.0)	4.6 ± 1.4 (4.7)	0.26	1.073 (0.771–1.493)	0.68
Rheumatoid factor (IU/mL)	162 ± 249 (69)	203 ± 242 (100)	0.42	1.001 (0.999-1.002)	0.43
MMP-3 (ng/mL)	178 ± 161 (123)	252 ± 211 (191)	0.03	1.002 (0.999-1.004)	0.17
Joint erosion score	62 ± 61 (36)	45 ± 46 (35)	0.41	0.993 (0.984-1.002)	0.12
Joint space narrowing score	45 ± 40 (32)	34 ± 35 (27)	0.28	0.992 (0.980–1.004)	0.21
Sharp score	107 ± 100 (68)	80 ± 80 (61)	0.36	0.996 (0.991–1.001)	0.15
Current PSL use (%)	38	55	0.14[a]	2.457 (0.964–6.259)	0.06
Current MTX use (%)	57	63	0.67[a]	1.464 (0.597–3.539)	0.41
Biologics use (%)	19	32	0.22[a]	2.718 (0.932–7.917)	0.07

CI confidence interval, *NA* not available, *CHD* congestive heart disease, *HDL/LDL* high/low-density lipoprotein, *HbA1c* glycated hemoglobin, *ESR* erythrocyte sedimentation rate, *DAS28* Disease Activity Score 28 joints, *MMP-3* matrix metalloproteinase-3, *PSL* prednisolone, *MTX* methotrexate

Unless indicated otherwise, data are presented as the mean ± standard deviation (SD), with the median given in parenthesis

Mann–Whitney U tests were used for comparisons between groups

[a] Fisher's two-tailed exact tests was used

(median 1,397 vs. 887 pg/mL, respectively; $P = 0.006$) (Fig. 2; Table 2). Since an elevated level of CRP has been recognized as a risk factor for atherosclerosis, we used logistic regression analysis to adjust for age, sex, and CRP. Even after adjusting for these three variables, elevation of the serum OPG level was still associated with carotid plaque ($P = 0.038$) (Table 2).

Discussion

The results of our study show that Japanese RA patients with carotid plaque had higher serum levels of OPG than patients without plaque. Established risk factors for atherosclerosis, disease activity of RA, joint damage, and corticosteroid use were not associated with the presence of

carotid plaque in our patients. However, an elevated serum OPG concentration was correlated with a higher prevalence of carotid plaque.

A major limitation of our study is that we did not include an age-matched control group without RA for comparison of the prevalence of carotid plaque. Ishizuka et al. [21] investigated the prevalence of carotid plaque by high-resolution B-mode carotid ultrasonography in 3,455 apparently healthy Japanese individuals who underwent regular health screening. Carotid plaque was detected in 21 % (725/3,455) of all subjects (mean age 57 ± 10 years) and in 13 % (145/1,127) of women (mean age 56 ± 10 years), while among our patient cohort we found plaque in 42 % of all RA patients and in 40 % of women. In another study, The Tokyo Health Service Association investigated the prevalence of carotid plaque by age strata in apparently healthy subjects undergoing a check-up [22]. The prevalence of carotid plaque among 242 healthy women who did not have RA was 5 % (2/40) in women younger than age 40 years, 5 % (5/99) for women in their 40s, 23 % (13/57) for women in their 50s, and 37 % (17/46) for women aged >60 years. Although a direct comparison could not be made between our RA patients and healthy subjects, the prevalence of carotid plaque was higher in our middle-aged Japanese patients with RA than in most healthy subjects. Roman et al. [3] assessed the prevalence of carotid atherosclerosis in RA patients ($n = 98$, mean age 48 ± 13 years) who were predominantly Caucasian and female (72 % Caucasian and 98 % female). They found that carotid plaque had a higher prevalence in the RA patients than in the controls (44 vs. 15 %, respectively) and that the prevalence of carotid plaque was 7 % in patients under the age of 40 years, 52 % for patients in their 40s, 52 % for patients in their 50s, and 80 % for patients aged >60 years. In other words, the prevalence of carotid atherosclerosis in Japanese patients with RA was higher than that in apparently healthy Japanese subjects, but lower than in Caucasian patients with RA.

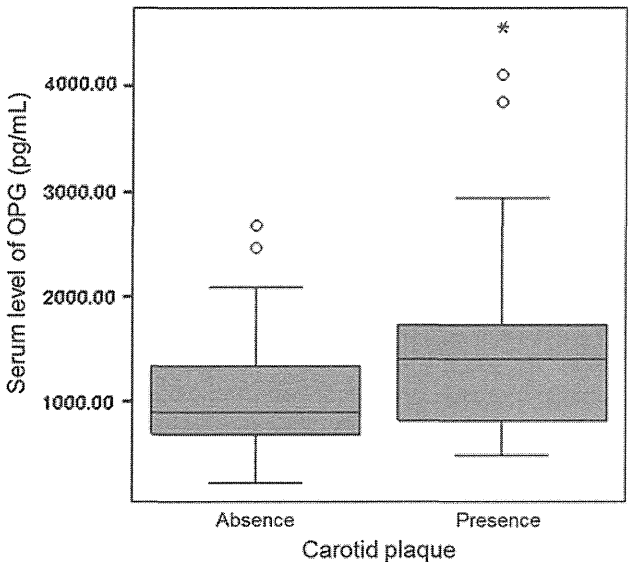

Fig. 2 Comparison of serum osteoprotegerin (*OPG*) concentration in patients with RA with and without carotid plaque, respectively. The serum OPG concentrations were significantly higher in patients with carotid plaque ($P = 0.006$)

Previous studies have demonstrated that traditional coronary risk factors do not fully explain the development of atherosclerosis in RA patients [2, 3]. Chronic inflammation and/or glucocorticoid therapy have been thought to play a role in the development of atherosclerosis in RA patients. However, we found that disease duration, disease activity, inflammation, and current glucocorticoid therapy were not associated with the presence of carotid plaque in our patients with RA. Compared with Caucasians, the lower incidence of cardiovascular disease in Japanese people is thought to depend on racial differences, a lower fat diet, and a lower frequency of traditional coronary risk factors. The increased prevalence of carotid atherosclerosis in Japanese RA patients may be explained by something other than traditional or known cardiovascular risk factors.

OPG is a soluble member of the tumor necrosis factor receptor superfamily that plays various roles in a wide range of biological activities, including bone metabolism, endocrine function, and immunity. OPG regulates osteoclastogenesis by binding with RANKL [23]. OPG has been suggested to have a role in the pathogenesis of RA and is also linked to atherosclerosis. It is highly expressed in RA synovial fibroblasts compared with fibroblasts from osteoarthritis and non-inflammatory joints [24]. OPG is also detected in human atherosclerotic plaque and it may influence plaque stability [25, 26]. The serum OPG concentration is a strong predictor of long-term mortality in patients with acute coronary syndrome that is independent

Table 2 Serum osteoprotegerin concentration in RA patients with and without carotid plaque, respectively

Serum osteoprotegerin	No plaque ($n = 53$)	Plaque ($n = 38$)	*P* value	Age and sex adjusted *P* value	Age, sex and CRP adjusted *P* value
Serum osteoprotegerin levels (pg/mL)	1,039 ± 545 (887)	1,551 ± 986 (1,397)	0.006	0.041	0.038

Mann–Whitney U tests were used for comparisons between groups. Logistic regression was used to assess age- and sex-adjusted *P* value or age-, sex-, and C-reactive protein-adjusted *P* value for presence of carotid plaque

Data are given as the mean ± SD, with the median in parenthesis

of conventional risk factors [27]. A prospective, population-based study demonstrated that the serum OPG concentration was associated with the severity and 10-year progression of carotid atherosclerosis [15], with an elevated OPG concentration being an independent risk factor for incident cardiovascular events and vascular mortality, but not for mortality due to nonvascular causes. Similar to its role in the general population, one of the important risk factors for accelerated atherosclerosis in RA may be elevation of the serum OPG level. It has been reported that increased serum concentrations of OPG were present in patients with RA and that the OPG concentration is independently associated with the coronary artery calcium score (which reflects coronary atherosclerosis) shown by electron beam computed tomography in patients with chronic RA (>10 years). The subjects of that study were predominantly Caucasian (approximately 85 %), and there was a positive association between OPG concentrations and disease activity of RA [28]. Our study shows that elevated serum OPG levels were also associated with carotid atherosclerosis in Japanese RA patients. Although the question of whether or not carotid plaque is a stronger predictor for coronary events is currently being strongly debated, a recent meta-analysis of 11 studies (54,336 patients) showed that carotid plaque is a stronger predictor of coronary events than the carotid intima-media thickness [29]. Evans et al. [30] reported that carotid plaque was a good predictor of the incidence of acute coronary syndrome in RA patients. These authors defined acute coronary syndrome as myocardial infarction, unstable angina, cardiac arrest, or death from ischemic heart disease and found that the incidence of new acute coronary events per 100 patient-years was 1.1 among RA patients without plaque, 2.5 among RA patients with unilateral plaque, and 4.3 among RA patients with bilateral plaque. An elevated OPG concentration may reflect an inflammatory process that promotes atherosclerotic plaque and leads to acute coronary syndrome in RA patients.

We found no association between the serum OPG concentration and joint destruction (bone erosions or joint narrowing) based on the van der Heijde-modified Sharp score in our RA patients. Since this study was cross-sectional, we could not investigate whether patients with elevated baseline OPG levels showed progressive bone and cartilage destruction over time. In another study, a cohort of 232 patients with RA was followed for 10 years to determine whether serum concentrations of cartilage and bone biomarkers could predict radiographic progression [31]. This long-term study found no association between the baseline serum level of OPG and radiographic progression. Thus, an increased serum OPG concentration seems to be an risk factor for accelerated atherosclerosis, but not for joint destruction, in patients with RA.

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Conflict of interest None.

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INTERNAL MEDICINE

□ CASE REPORT □

Immunopathological Analysis of Erdheim-Chester Disease with Massive Ascites

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Abstract

We treated a 77-year-old woman with pleural and pericardial effusion and ascites. Initially, collagen vascular disease was suspected due to the presence of anti-centromere antibodies and suspected complication of pulmonary arterial hypertension. However, soft-tissue abnormalities surrounding the bilateral kidneys detected on computed tomography (CT) and symmetrical lesions of the long bones detected on bone scintigraphy made us consider a diagnosis of Erdheim-Chester disease (ECD), which is a rare form of histiocytosis. We immunochemically analyzed the cells derived from the ascites in detail and confirmed the diagnosis. Immunocytochemical analyses may therefore help to achieve a better understanding of the pathogenesis of this rare disease.

Key words: Erdheim-Chester disease, histiocytosis, flow cytometry, interferon

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Introduction

Erdheim-Chester disease (ECD) is a rare form of histiocytosis of unknown origin (1). It is distinguished from Langerhans histiocytosis (LCH) in that the abnormal histiocytes of ECD are positive for CD68 and negative for CD1a and S-100 proteins. ECD is a multisystem disease that primarily affects the skeletal system but also involves the central nervous system, cardiovascular system, hypothalamus-pituitary system, lungs, kidneys, retroperitoneum and orbits. It remains unclear whether ECD is a malignant or reactive inflammatory disease. Since the first report in 1930, less than 200 cases of ECD had been reported as of the end of 2007 (2). Since then, however, more than 200 cases have been reported in less than four years (1). The increased number of reported cases is probably due to the fact that awareness of the disease has become more widespread in recent years. Hopefully, accumulating data related to ECD will increase our understanding of the pathogenesis of this disease and also help to establish better treatment strategies.

Case Report

A 77-year-old woman was admitted to this hospital with dyspnea, palpitations and ascites. One year before admission, she developed dyspnea on exertion. Pericardial effusion was detected at a nearby hospital and a diuretic was prescribed. The dyspnea persisted and peripheral edema newly developed. She was admitted to another hospital, where pericardiocentesis was performed. The pericardial effusion was reported to be bloody and exudative. The cytological grade was Class I, and no bacteria were detected, including *Mycobacterium tuberculosis.*

The amount of pericardial effusion decreased spontaneously and she was discharged on the 16th day of hospitalization. Within one month, however, abdominal bloating developed and she was referred to our hospital. As the antinuclear antibody test was positive at 1:640, she was referred to this department.

On examination, the patient's temperature was 37.0℃, pulse was 78 beats per minute and blood pressure was 170/80 mmHg. Her weight was 54.0 kg and height was 143.4

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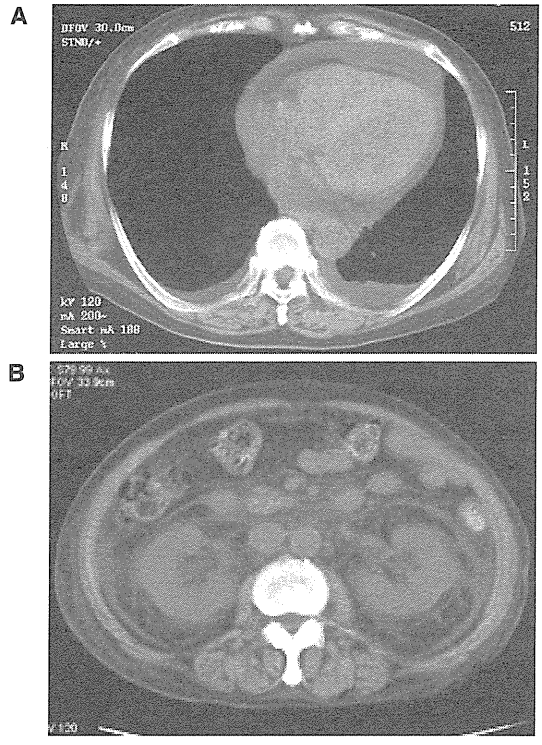

Figure 1. Chest and abdominal CT scans. (A) Pleural effusion and pericardial effusion were evident. (B) Bilateral and symmetrical perirenal soft tissue lesions were detected ("hairy kidney" appearance). A small amount of ascites was also present.

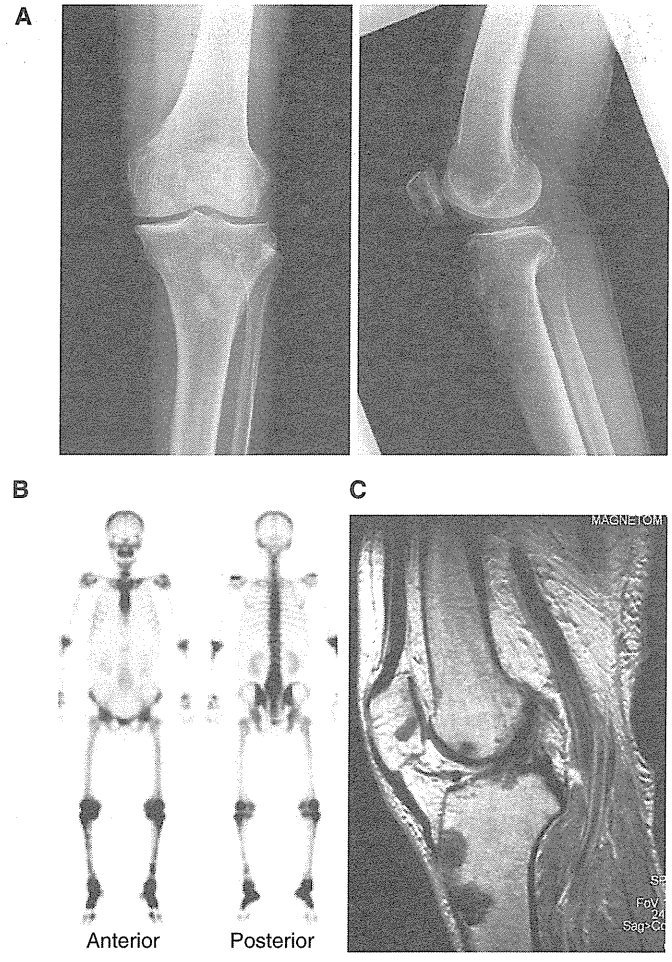

Figure 2. Skeletal imaging of the legs. (A) X-ray showed the presence of osteosclerotic lesions in the tibia. (B) Symmetrically increased osteoblastic activity primarily in the lower limbs was detected on bone scintigraphy. The diaphyses and metaphyses of the femurs and the tibiae were primarily affected, whereas the mid-diaphyses and epiphyses were spared. (C) MR imaging revealed bone lesions at the same positions as those shown in (A).

cm. She did not have exophthalmos. Her chest sounds were normal. Her abdomen was distended. Mild abdominal tenderness was observed; however, no rebound tenderness was present and her bowel sounds were normal. No edema of the lower extremities or skin rashes were observed. The leukocyte count was $5.86\times10^3/\mu L$ (72.3% neutrophils, 19.1% lymphocytes), the hemoglobin level was 10.1 g/dL and the platelet count was $176\times10^3/\mu L$. The C-reactive protein (CRP) level was 0.18 mg/dL. The patient's liver function was normal; however, the creatinine level was 1.32 mg/dL, the blood urea nitrogen level was 23 mg/dL and the potassium level was 5.4 mEq/L, suggesting the presence of chronic kidney disease. The free T3 and free T4 levels were low (1.53 pg/mL and 0.83 pg/mL, respectively), while the thyroid stimulating hormone (TSH) level was high (6.64 μIU/mL), indicating hypothyroidism. The anti-nuclear antibodies were of the discrete speckled type. As expected, the patient was positive for anti-centromere antibodies (INDEX: 128; reference range: 0-9.99); however, no other disease-specific antibodies were detected, including antibodies to double-stranded deoxyribonucleic acid (DNA), Sm, ribonucleoprotein (RNP) or Scl-70. A urinalysis revealed the presence of slight proteinuria (45 mg/dL), scant red blood cells (1-4/high power field (HPF)), hyaline casts (50-99/whole field (WF)) and granular casts (5-9/WF).

Chest X-ray revealed cardiac enlargement and computed tomography (CT) revealed bilateral pleural effusions, pericardial effusion and ascites (Fig. 1). Characteristically, soft-tissue abnormalities surrounding the circumference of the bilateral kidneys were observed (Fig. 1B). The right ventricular systolic pressure (RVSP) was estimated to be 60.4 mmHg using echocardiography, which suggested the presence of pulmonary arterial hypertension (PAH).

A diagnosis of systemic sclerosis was initially suspected; however, no dermal sclerosis was apparent. Diagnostic thoracentesis was performed. The levels of protein and lactate dehydrogenase (LDH) in the pleural effusion were 3.6 g/dL and 53 IU/L and those in the plasma were 6.2 g/dL and 84 IU/L, respectively. In addition, the specific gravity and white blood cell concentration were both relatively high (1.027 and 978/μL, respectively). Therefore, the effusion was considered to consist of exudates. Although a diagnosis of systemic sclerosis was not confirmed, the presence of inflammation and the negative test results for infection led us to decide to treat the patient with 30 mg of prednisolone per day. The patient's hypothyroidism was treated with synthetic

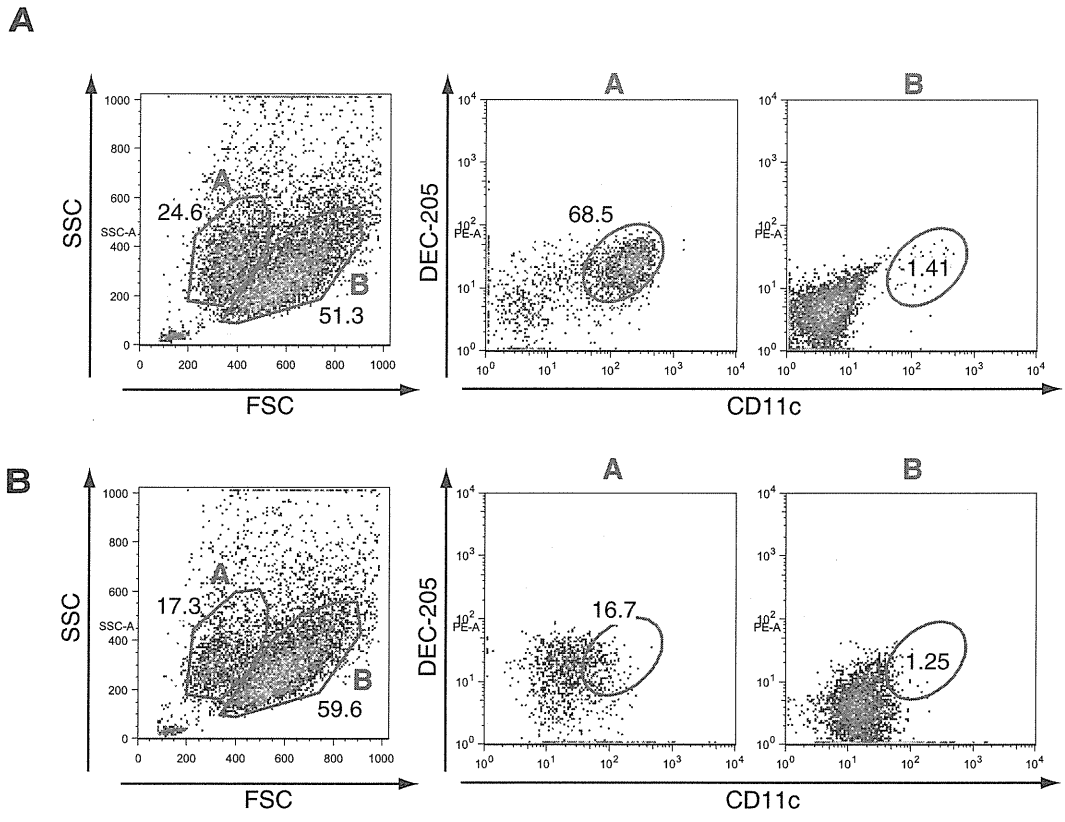

Figure 3. Flow cytometric analysis of the cells in the ascites. (A) More than one-half of the cells in gate A were positive for both CD11c and DEC-205 antigens, whereas those in gate B were double-negative for the antigens. Cells cultivated *in vitro* for seven days were used for the analysis. Dead cells were excluded with 7-aminoactinomycin D staining. (B) *In vitro* cultured cells were analyzed 15 hours after treatment with 10 ng/mL IFN-α. Note that the number of cells in gate A decreased significantly. Moreover, most of the cells became negative for CD11c and DEC-205 antigens.

thyroid hormone (125 μg/day). The abdominal distension improved after treatment with the corticosteroid was initiated, and the patient was discharged approximately two months later. She received follow-up care as an outpatient of this hospital for one month; however, during the course of tapering the dose of prednisolone, dyspnea on exertion, abdominal distension and edema of the lower extremities again worsened. She was therefore readmitted to this department for further evaluation. Taking into account the possibility that the abnormal soft tissue around the kidneys represented retroperitoneal fibrosis, we performed a peritoneal biopsy. Only non-specific findings were observed such as mild fibrosis and congestion (data not shown). Immunostaining revealed no signs of IgG4 deposition or IgG4-producing plasmacytes, excluding the diagnosis of IgG4 syndrome. Unexpectedly, four months after the initial assessment, the RVSP estimated with echocardiography was 38 mmHg, although we did not use any medications for PAH, casting doubt on the presence of PAH. To relieve the symptoms of abdominal fullness, it was necessary to perform abdominal paracentesis one to two times per week. Repeated cytological examinations always revealed Class II and the presence of histiocytes and a small number of activated mesothelial cells. These findings, along with the peculiar soft tissue abnormality around the kidneys, led us to suspect a diagnosis of ECD. Bone scintigraphy demonstrated a symmetrically increased osteoblastic activity in the lower limbs primarily affecting the diaphyses and metaphyses of the femurs and the tibiae but sparing the mid-diaphyses and epiphyses. These are the almost pathognomonic findings of ECD (2) (Fig. 2B). X-rays of the knees revealed sclerotic bone lesions and magnetic resonance (MR) imaging revealed multiple bone lesions with irregular boundaries (Fig. 2A, C). Although ECD was strongly suspected, the histological evidence was lacking. The patient refused to undergo a biopsy of the bone lesions. As a second-best option, we immunologically analyzed the cells derived from the ascites.

In a flow cytometric analysis (Fig. 3A), two distinct populations (A and B) were gated using forward scatter (FSC) and side scatter (SSC). The cells in gate A were found to be double-positive for CD11c and dendritic and epithelial cells, 205 kDa (DEC-205) antigens, while those in gate B were found to be negative for both antigens. We next performed immunocytochemistry of the pellet of the ascites cells (Fig. 4 and Table). More than 50% of the cells were positive for CD68 and none of the cells were positive for S-100 proteins. The cells were also diffusely positive for DEC-205 and partially positive for dendritic cell-specific in-

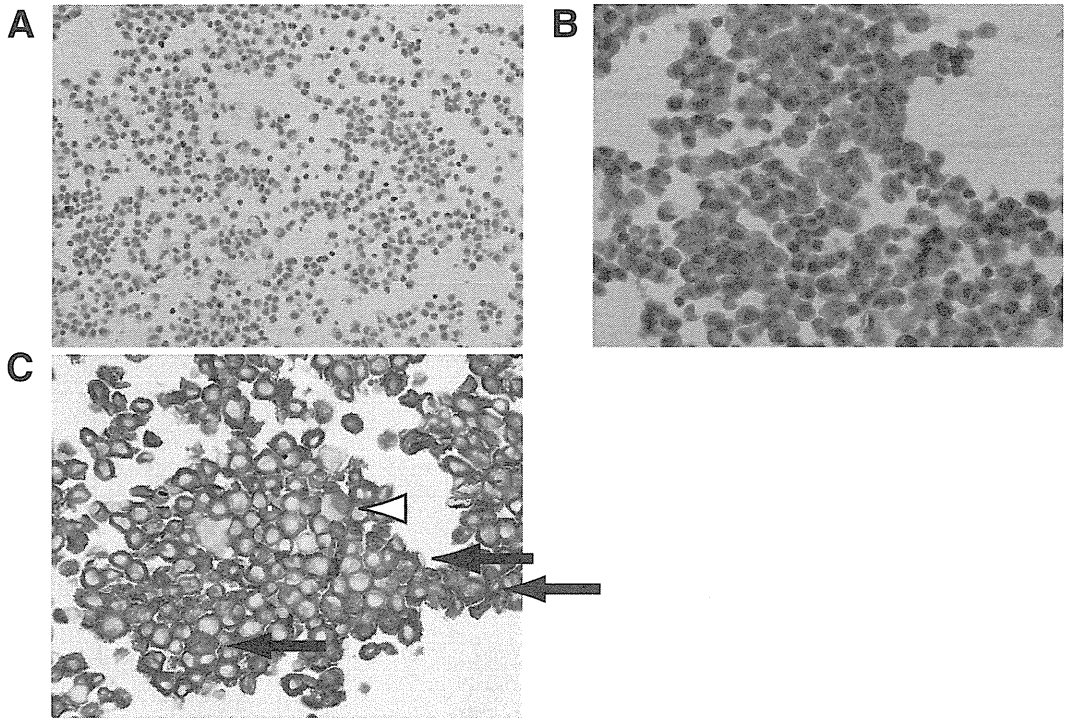

Figure 4. Immunostaining of cell pellets derived from the ascites. (A) Most of the cells were S-100-protein-negative. Counterstained with Hematoxylin and Eosin staining, ×40. (B) Cells were diffusely positive for DEC-205. Counterstained with Hematoxylin and Eosin staining, ×400. (C) Double staining for CD163 (brown) and Ki-67 (red). Some of the CD163-positive cells were also Ki-67-positive (black arrows), indicating that the cells were proliferating. A white arrowhead indicates a cell that was positive for Ki-67 and negative for CD163. Uncounterstained with Hematoxylin and Eosin staining, ×400.

tercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). Interestingly, double staining for CD163 and Ki-67 revealed almost all of the cells to be positive for CD163 and 5-10% of them were also positive for Ki-67, thus indicating that the cells were still proliferating.

Two courses of steroid pulse (methylprednisolone (mPSL) 500 mg ×3) were administered over an interval of one month, and, during this period, oral mPSL was reduced from 32 mg/day to 18 mg/day. Thereafter, the amount of ascites decreased somewhat and it was no longer necessary to perform abdominocentesis. Although there is no standard treatment for ECD, several reports have indicated the efficacy of interferon (IFN)-α (3-5). We therefore attempted to treat the patient with IFN-α; however, the treatment had to be discontinued due to fever, appetite loss and deterioration of renal function.

The patient and her family did not wish for any further vigorous therapy to be administered; therefore, she was transferred to a nearby hospital and treated with oral mPSL.

Discussion

We encountered a case of ECD with ascites. The "hairy kidney" appearance of the kidneys caused by bilateral infiltration of the perirenal and posterior pararenal spaces was highly indicative of the disease (6), while bone scintigraphy showed the pathognomonic appearance of ECD skeletal involvement (2). Osteosclerotic lesions detected on X-rays and bone cyst-like lesions detected on MR imaging were also compatible with a diagnosis of ECD (7). A diagnosis of ECD is usually confirmed with typical pathological findings: xanthogranulomas infiltrated by foamy histiocytes that are positive for CD68 and negative for CD1a (8). As we did not obtain the consent of the patient to perform a bone biopsy, we confirmed the diagnosis based on an immunocytological analysis of the cells derived from the ascites. It was obvious from the analysis that the abnormal histiocytes were of monocyte/macrophage origin; however, negative staining for CD15 suggests that the cells had lost the characteristics of monocytes/macrophages. Moreover, the findings of flow cytometry that the cells were positive for DEC-205, DC-SIGN and CD11c suggest that the cells had developed into interstitial dendritic cells. Interestingly, some of the CD68-positive cells were also positive for Ki-67, thus indicating that the cells were still proliferating, which is unusual for differentiated cells.

Initially, we suspected a diagnosis of systemic sclerosis, even though the patient had no signs of dermal sclerosis, because the anti-centromere antibody test result was positive and the RVSP estimated on echocardiography was high. However, repeated echocardiography showed decreases in estimated RVSP to the level of 38 mmHg over four months

Table. Results of the Immunocytochemical Analysis of Cell Pellets Derived from Ascites

Antibody (clone)	Ig subclass	Source	Cells	Results in this case
CD163 (10D6)	Mouse IgG1	Novocastra, Newcastle upon Tyne, UK	Monocytes/macrophages	Diffusely positive
CD68 (KP-1)	Mouse IgG1	DAKO, Carpinteria, CA	Monocytes /macrophages	Positive (>50%)
Anti-macrophage (LN5)	Mouse IgM, κ	Invitrogen, Camarillo, CA	Monocytes /macrophages	Positive (>50%)
Vimentin (V9)	Mouse IgG1	Santa Cruz, Delaware Avenue, CA	Stromal cells	Diffusely positive
DEC-205 (CD205) (11A10)	Mouse IgG1	Novocastra	Dendritic cells (macrophages)	Diffusely positive
DC-SIGN (CD209)	Rabbit IgG	Santa Cruz	Dendritic cells (macrophages)	Partially positive
CD15 (Leu M1)	Mouse IgG1, κ	Abcam, Cambridge, UK	Myelocytes /monocytes	Negative
EMA (E29)	Mouse IgG2a, κ	DAKO	Epithelial cells	Negative
Calretinin (DAK-Calret 1)	Mouse IgG1, κ	DAKO	Mesothelial cells	Negative (the few positive cells were methothelial cells)
CD1a (O10)	Mouse IgG1, κ	Immunotech, Marseille, France	Langerhans cells	Negative
S-100 protein	Rabbit, heterologous	Nichirei, Tokyo, Japan	Langerhans cells	Negative
Ki-67 (MIB-1)	Mouse IgG1	Immunotech	Proliferating cells	5-10% positive

and to 27.4 mmHg over nine months despite the fact that the patient did not receive any medications for PAH. Therefore, we doubted the presence of PAH. Performing right heart catheterization would have been necessary to confirm the diagnosis (9); however, it was not performed. Considering that the patient did not show any signs of visceral involvement, such as esophageal hypomotility, small bowel hypomotility or pulmonary interstitial fibrosis and did not have a history of Raynaud's phenomenon, we now believe that the coexistence of "systemic sclerosis sine scleroderma" (10) was unlikely. The positive result for the anti-centromere antibody test may thus have been non-specific.

Regarding the treatment of ECD, corticosteroids are known to have a limited effect. We did observe, however, that oral prednisolone (30 mg/day) decreased the amount of ascites somewhat for the time being. Moreover, two courses of steroid pulse therapy seemed to inhibit increases in the amount of ascites, rendering abdominocentesis unnecessary. The estimated systolic right ventricular pressure also normalized after treatment, although the causal linkage was not clear. On the other hand, a second abdominal CT scan repeated after approximately six months revealed almost no changes in the soft tissue abnormality around the kidneys.

Recently, treatment with IFN-α has been shown to improve the survival of ECD patients (3). Unfortunately, high fever, appetite loss and renal function deterioration, most likely due to dehydration, did not allow us to continue to administer IFN-α to our patient. The mechanisms by which IFN-α exerts therapeutic effects in this disease have not yet been clarified; however, i) maturation of abnormal cells, ii) immune-mediated destruction of cells and iii) anti-proliferative effects of IFN-α have been suggested. We added recombinant IFN-α (10 ng/mL) to the cells derived from the ascites *in vitro* and attempted to analyze the cells with flow cytometry 15 hours later. To our surprise, the cells in gate A (Fig. 3) significantly decreased in number and most of the remaining cells were found to be double-negative for CD11c and DEC-205. This may indicate that IFN-α specifically induces death in abnormal cells. We were unable to confirm whether the observed cell death was due to apoptosis because we were unable to obtain fresh ascites after two courses of steroid pulse therapy.

To the best of our knowledge, this is the first report of ECD in which abnormal cells were analyzed using both flow cytometry and detailed immunocytochemistry. The cells derived from the ascites were safely obtained and proved to be quite useful for confirming the diagnosis. Moreover, multi-staining procedures and the analysis of the expression of various molecules in abnormal cells may also be helpful for elucidating the pathogenesis of this rare disease.

The authors state that they have no Conflict of Interest (COI).

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Special Report

Prevention of hepatitis B virus reactivation in patients receiving immunosuppressive therapy or chemotherapy

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With the increasing use of potent immunosuppressive therapy, reactivation of hepatitis B virus (HBV) in endemic regions is becoming a clinical problem requiring special attention. A recent annual nationwide survey clarified that HBV reactivation related to immunosuppressive therapy has been increasing in patients with malignant lymphoma, other hematological malignancies, oncological or rheumatological disease. In the survey, rituximab plus steroid-containing chemotherapy was identified as a risk factor for HBV reactivation in hepatitis B surface antigen (HBsAg) negative patients with malignant lymphoma. In this setting, HBV reactivation resulted in fatal fulminant hepatitis regardless of the treatment of nucleoside analog. The Intractable Hepatobiliary Disease Study Group and the Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis jointly developed guidelines for preventing HBV reactivation. The essential features of the guideline are as follows. All patients should be screened for HBsAg by a sensitive method before the start of immunosuppressive therapy. Second, hepatitis B core antigen (HBcAb) and hepatitis B surface antibody (HBsAb) testing should be performed in HBsAg negative patients, especially those receiving intensive immunosuppressive therapy. Prophylaxis with nucleoside analogs is essential for preventing HBV reactivation in HBsAg positive patients. In contrast, HBsAg negative with HBcAb and/or HBsAb positive patients should be monitored monthly for an increase in serum HBV DNA during and 12 months after completion of chemotherapy. Nucleoside analogs should be administrated immediately when HBV DNA becomes positive during this period. This strategy facilitates commencement of nucleoside analogs at an early stage of HBV reactivation and results in prevention of severe hepatitis.

Key words: de novo hepatitis, fulminant hepatitis, hepatitis B virus reactivation, immunosuppressive therapy, rituximab

INTRODUCTION

HEPATITIS B VIRUS (HBV) is the most frequently identified agent that causes acute or chronic hepatitis in Eastern Asia. In Japan, approximately 970 000 people are infected with HBV, as estimated by hepatitis B surface antigen (HBsAg) testing in blood donors.[1] Chronic HBV carriers have a 15–40% lifetime risk of developing serious complications of chronic liver disease.[2] However, most carriers remain clinically silent for extended periods and some carriers will lose HBsAg over a long lifetime. In adults, most acute HBV infections are self-limited, and recovery occurs naturally. Seroconversion from acute HBV infection with HBsAg to antibody to hepatitis B surface antibody (HBsAb) is believed to represent viral clearance. Clearance of HBsAg and appearance of antibody to hepatitis B core antibody (HBcAb) with or without HBsAb provides evidence of resolved infection in patients. However, with the advent of sensitive polymerase chain reaction techniques for detecting HBV DNA in serum and liver, it has been shown that most HBsAb/HBcAb positive patients have HBV DNA in the liver and/or serum. It is estimated that 2 billion people worldwide have been infected with HBV.[3] In Japan, it is reported that 23.2% of blood donors are positive for HBcAb and/or HBsAb.[4]

Reactivation of HBV is a well-recognized complication in HBsAg positive patients who are undergoing immunosuppressive chemotherapy for cancer. The clinical manifestation ranges from subclinical hepatitis to severe, potentially fatal fulminant hepatitis. In this decade, HBV reactivation has been observed in patients

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with resolved infection (HBsAg negative and HBcAb and/or HBsAb positive) who have undergone intensive immunosuppressive chemotherapy such as rituximab plus steroid-containing chemotherapy. Previous reports have clarified that this combination therapy can lead to fulminant hepatitis and even death. For this reason, clinicians need to be aware of HBV reactivation not only in patients with current infection but also in those with resolved infection who are undergoing intensive immunosuppressive therapy.

This report summarizes the important issues related to HBV reactivation and suggests a guideline for preventing this condition in the clinical setting.

HBV REACTIVATION IN HBsAg POSITIVE CARRIERS

LIFE IN HBV carriers can generally be divided into four distinct phases: (i) immune tolerance phase; (ii) immune active phase; (iii) low-replication phase; and (iv) resolved phase. Inactive carriers in the low-replication phase are frequently associated with antibodies to hepatitis B e antigen (anti-HBe) seroconversion with a low viral load (<4.0 log copies/mL).[5] Sustained host immune control over viral replication in the low-replication phase may lead to HBsAg seroclearance. During the low-replication phase, 20–30% of patients may develop spontaneous HBV reactivation.[6] Patients with chronic HBV infection receiving immunosuppressive chemotherapy usually have impaired host immunity that may allow active HBV replication to occur. Following the completion of therapy, restoration of host immunity against HBV occurs, resulting in extensive cytotoxic T-cell-mediated lysis of the infected hepatocytes and clinical hepatitis flares. Some patients experience severe hepatitis with HBV reactivation, with fatality rates ranging 5–40%.[7–10]

The risk of HBV reactivation is mainly related to the underlying disease, intent of the immunosuppression and HBV replicative state. The risk is particularly high in patients with lymphoma.[11,12] Patients with other hematological malignancy such as multiple myeloma and B-cell chronic lymphocytic leukemia are also at risk.[13] Patients receiving intensive cytoreductive therapy and high-dose chemotherapy are highly susceptible to HBV reactivation. In non-hematological tumors, the rate of HBV reactivation is high in patients with breast cancer.[14,15] HBV reactivation can also occur in patients with non-malignant disease, such as rheumatological disease and collagen disease.[13]

The use of chemotherapy regimens containing corticosteroids or anthracycline increases the risk of HBV reactivation.[7,11,12,16,17] HBV DNA contains a glucocorticoid responsive element that has been suggested to facilitate HBV replication.[18,19] Anthracycline has also been demonstrated *in vitro* to stimulate HBV DNA replication.[20] Recently, the use of the anti-CD20 monoclonal antibody, rituximab, appears to be an independent risk factor of HBV reactivation.[21] This agent causes profound and long-lasting immunosuppression, reflecting a decrease in CD20 cells and HBsAb titer.[22] There are many reports of HBV reactivation following the use of rituximab as monotherapy[23] or in combination with other types of chemotherapy.[24–27]

REACTIVATION IN HBsAg NEGATIVE PATIENTS WITH HBcAb AND/OR HBsAb

IN PATIENTS WITH resolved HBV infection (HBsAg negative, HBsAb and/or HBcAb positive), HBV replication has been shown to persist in the liver and in peripheral blood mononuclear cells.[28,29] Cellular and humoral immune surveillance suppresses viral replication.[30,31] In the life cycle of HBV replication, covalently closed circular DNA (cccDNA) is formed in the nuclei of infected hepatocytes. cccDNA is the main template for the transcription of viral mRNA and has been shown to persist in the liver.[21] With impairment of the host defense system, cccDNA can evade host immunity and actively replicate again. This scenario of HBV reactivation in patients with resolved infection of so-called "de novo hepatitis B" appears to be a new clinical issue.

Hepatitis B virus reactivation has been reported in this setting after transplantation and allogenic and autologous hematopoietic stem-cell transplantation with the reappearance of HBsAg.[32–34] In recent years, the incorporation of rituximab with standard chemotherapy is associated with HBV reactivation in patients with non-Hodgkin's lymphoid malignancies. Hui *et al.* reported that the incidence of HBV reactivation with a combination of rituximab plus steroids was higher (12.2%, 6/49) compared with other combinations of therapy (1.0%, 2/195).[35] Recently, Yeo *et al.* reported that 23.8% of HBsAg negative/HBcAb positive lymphoma patients receiving rituximab plus steroid combination therapy developed HBV reactivation.[27] It is notable that there are a number of case reports of fatal hepatitis in HBcAb positive patients who received rituximab-containing chemotherapy for lymphoma.[24–27,36] Umemura *et al.* reported that the rate of fulminant hepatitis and

Table 1 Causes of HBV-related fulminant hepatitis and late-onset hepatic failure (LOHF)

	Total	Years					
		2004	2005	2006	2007	2008	2009
All patients	194 (9)	26 (2)	42 (2)	27	37 (1)	23 (2)	39 (2)
Transient infection	91 (1)	12 (1)	23	13	17	11	15
Carrier	72 (7)	9 (1)	11 (1)	9	14 (1)	11 (2)	18 (2)
Inactive carrier	35 (1)	6	7	3	5	3	11 (1)
Reactivation (inactive carrier)	20 (5)	2	3 (1)	1	4 (1)	5 (2)	5 (1)
Reactivation (resolved infection)	17 (1)	1 (1)	1	5	5	3	2
Undetermined	31 (1)	5	8 (1)	5	6	1	6

Data shown indicate the number of patients, and those in parentheses indicate the number of patients with LOHF.

mortality following de novo hepatitis B is high compared with acute hepatitis B in Japan.[37]

FULMINANT HEPATITIS CAUSED BY HBV REACTIVATION IN JAPAN

THE INTRACTABLE HEPATOBILIARY Diseases Study Group in Japan annually performs a nationwide survey of patients with fulminant hepatitis and late-onset hepatic failure (LOHF).[38] A recent annual nationwide survey from 2004 to 2009 revealed that HBV infection prevailed in 39.8% (194/488) of patients with fulminant hepatitis and LOHF. It is noteworthy that 19.1% (37/194) of HBV related-hepatitis was caused by HBV reactivation following immunosuppressive therapy or chemotherapy (Table 1). Furthermore, almost half of these patients have evidence of HBV reactivation from resolved infection (HBsAg negative before the start of therapy and HBsAg positive and HBcAb and/or HBsAb positive at the onset of hepatitis). The total number of patients with HBV reactivation has been increasing since 2004. We first compared the clinical features of 37 patients with HBV reactivation with those of transient infection and those with acute exacerbation (Table 2). The age of the patients was higher in the HBV reactivation group than that in the transient infection

Table 2 Clinical characteristics of patients with hepatitis B virus (HBV) reactivation, compared with those of patients with transient infection and HBV carriers who developed spontaneous acute exacerbation

	Transient infection ($n = 91$)	Acute exacerbation in HBV carriers ($n = 35$)	HBV reactivation ($n = 37$)
Age, years, median (range)	46 (17–72)	53 (15–89)	64 (29–86)**††
Male/female	58/33	23/12	22/15
Disease types (F-A/F-SA/LOHF)	80/10/1	14/20/1**	4/27/6**††
Prognosis (alive/died/LT)	40/36/15	7/18/10*	2/33/2**††
ALT, IU/L (mean ± SD)	4207 ± 2725	989 ± 1183**	902 ± 1380**
Total bilirubin, mg/dL (mean ± SD)	10.8 ± 8.2	15.2 ± 10.3*	15.8 ± 7.7**
Prothrombin time (%), median (range)	18.4 (3.1–58.6)	24.9 (2.2–58.1)**	29.8 (8.0–48.0)**
HBV DNA level, log copies/mL (mean ± SD)	5.6 ± 1.4	6.3 ± 1.7*	7.2 ± 1.4**†
Treatment			
Lamivudine	57 (63)	19 (54)	22 (59)
Entecavir	29 (32)	16 (46)	18 (49)
Interferon	28 (31)	11 (31)	11 (30)

Unless otherwise indicated, data indicate the number of patients, and those in parenthesis indicate percentages of patients.
Laboratory data are at the onset of hepatic encephalopathy of coma grade greater than II. HBV DNA levels are at the onset of hepatitis.
Significant difference among group was assessed by Student's *t*-test, Mann–Whitney *U*-test and χ^2-test.
Values significantly difference from patients with transient infection; $^*P < 0.05$, $^{**}P < 0.01$.
Values significantly different from patients with acute exacerbation in HBV carriers; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$.
ALT, alanine aminotransferase; F-A, acute type fulminant hepatitis; F-SA, subacute type fulminant hepatitis; LOHF, late-onset hepatic failure; LT, liver transplantation; SD, standard deviation.

and acute exacerbation groups. There was a tendency for the reactivation group to show clinical manifestation of the subacute type or LOHF. The reactivation group had lower alanine aminotransferase (ALT) levels and higher bilirubin and HBV DNA levels. Of the 37 cases of HBV reactivation, 33 (89%) resulted in liver-related death, two (5%) survived and two(5%) received living-donor liver transplantation. We then compared the clinical features of the 20 patients with HBV reactivation in HBsAg positive carrier status with those of the 17 patients with HBsAg negative resolved HBV infection status (Table 3). The resolved infection group was older than the carrier group. Most of the resolved infection group showed clinical manifestation as subacute type. The resolved infection group had lower ALT levels and higher bilirubin levels than those in the carrier group. It is noteworthy that all patients with resolved infection who developed HBV reactivation died despite nucleoside analog treatment. Concerning underlying disease, non-Hodgkin's lymphoma or mucosa-associated lymphoid tissue lymphoma was most prevalent in 50% of the carrier group and in 76% of the resolved infection group, respectively. In the carrier group, there were patients with oncological, rheumatological or collagen disease. HBV reactivation occurred more frequently after immunosuppressive therapy in patients with resolved infection, as previously reported.[39] Rituximab plus steroids combination chemotherapy was administrated to 35% of patients in the carrier group and to 59% of patients in the resolved infection group, respectively. Corticosteroid was used as monotherapy or in combination therapy in approximately three quarters of both groups. Methotrexate and anthracycline antitumor agent were given in 10% in the carrier group.

PREVENTION OF HBV REACTIVATION FOLLOWING IMMUNOSUPPRESSIVE CHEMOTHERAPY

HBsAg positive patients

BECAUSE VIRAL REPLICATION precedes clinical evidence of hepatitis, it is efficacious to use nucleoside analogs in a prophylactic manner before the start of chemotherapy. Previous retrospective and prospective studies have shown that the risk of HBV reactivation can be greatly reduced by the use of prophylactic nucleoside analog therapy for susceptible patients.[15,40–43] In Japan, currently, there are three oral nucleoside analogs approved for the treatment of chronic hepatitis B (lamivudine, adefovir and entecavir). Concerning lamivudine, the drug has proven efficacy and safety in preventing HBV reactivation related to chemotherapy. However, a major problem with its prolonged use is the possibility of viral breakthrough following the emergence of treatment-resistant HBV variants with YMDD mutations.[44] Given their high potency and extremely low rates of drug resistance, new generation oral nucleoside analogs, such as entecavir or tenofovir, are anticipated to be effective for HBV reactivation. The incidence of entecavir resistance in nucleos(t)ide analog-naive patients is reported to be 1.2% at 3 years.[45–47] A recent report demonstrated that entecavir is effective in the prevention of HBV reactivation in cancer patients.[48,49]

The American Association for the Study of Liver Disease (AASLD) guidelines recommend that if the anticipated duration of treatment is less than 1 year and baseline serum HBV DNA is not detectable, lamivudine or telbivudine are desirable and in other cases, entecavir or tenofovir are desirable.[50] The European Association for the Study of the Liver (EASL) guidelines also recommend the use of lamivudine for patients with low HBV DNA and entecavir or tenofovir for patients with high HBV DNA.[51] A consensus of the Japan Society of Hepatology recommends the use of entecavir as the first-line drug when patients with chronic hepatitis B are treated.[52]

Although the optimal time point for the initiation of antiviral prophylaxis has not been clearly established, nucleoside analogs should ideally be started as early as possible before chemotherapy.[53] This strategy can prevent any increase in viral replication, reduce the likelihood of drug resistance, allow chemotherapy to be completed and minimize the risk of hepatitis flare-up once chemotherapy is stopped.

Another concern with the use of lamivudine has been the occurrence of hepatic flares upon cessation of the antiviral compound. The optimal duration of antiviral prophylaxis in HBsAg carriers receiving immunosuppressive chemotherapy has only partly been clarified and is under active investigation. Several cases of HBV reactivation and even fatal fulminant hepatitis have been reported when lamivudine was stopped less than 3 months after the completion of chemotherapy.[54] AASLD guidelines recommend that prophylaxis is discontinued 6 months after completion of chemotherapy in patients with baseline HBV DNA of less than 2000 IU/mL, otherwise prophylaxis continues. EASL guidelines recommend the same treatment as that for AASLD guidelines for 12 months after completion of chemotherapy.