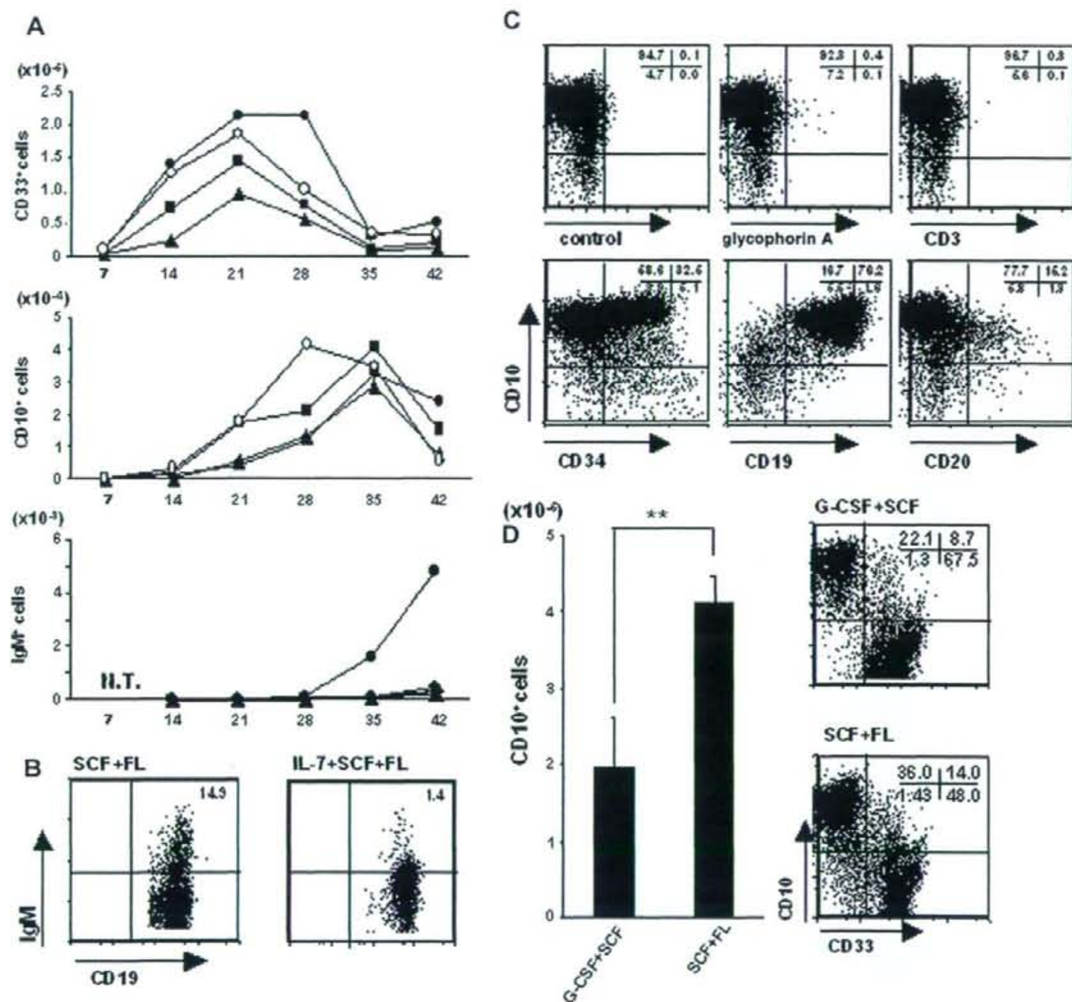


Figure 2. Combination of cytokines enhances human B-lymphocyte production in cocultures of cord blood (CB) CD34⁺ cells on human mesenchymal stem cells (hMSC). (A) Purified CB CD34⁺ cells (2000 cells/well) were cultured on hMSC in combinations of 10 ng/mL stem cell factor (SCF) + 5 ng/mL Flt3-ligand (FL) (closed circle), 5 ng/mL FL + 5 ng/mL interleukin (IL)-7 (closed triangle), 5 ng/mL IL-7 + 10 ng/mL SCF (closed square), 10 ng/mL SCF + 5 ng/mL FL + 5 ng/mL IL-7 (open circle) for 6 weeks. The cultured floating cells were collected, and numbers of the generated CD33⁺ cells (upper panel), CD10⁺ cells (middle panel), and IgM⁺ cells (lower panel) were estimated weekly. Similar results were obtained in three independent experiments. (B) Purified CB CD34⁺ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF + 5 ng/mL FL or 10 ng/mL SCF + 5 ng/mL FL + 5 ng/mL IL-7, respectively. At day 42, the generated cells were stained with fluorescein isothiocyanate (FITC)-IgM and phycoerythrin (PE)-CD19, and analyzed with flow cytometry. Similar results were obtained in three independent experiments. (C) Purified CB CD34⁺ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL. The generated cells were stained with FITC-CD33 and allophycocyanin (APC)-CD10 as well as the indicated PE-conjugated antibody (Ab), and analyzed with flow cytometry at day 40. Isotype-matched Abs were used as negative controls. Similar results were obtained in three independent experiments. (D) Purified CB CD34⁺ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF + 5 ng/mL FL or 10 ng/mL SCF + 10 ng/mL G-CSF. Numbers of the generated CD10⁺ cells were analyzed at 4 weeks of the cocultures. Data are shown as mean ± standard deviation in triplicated samples. Statistically differences from control values are shown with two ($p < 0.01$) asterisks. One representative flow cytometry data for CD10 and CD33 expression was also shown. Similar results were obtained in two independent experiments. NT = not tested.

CD10 Ab recognizes neutral endopeptidase, a 100-kD type II transmembrane glycoprotein, which is referred to as the common acute lymphoblastic leukemia antigen. CD10 antigen is expressed on some subsets of B- and T-lymphoid progenitors and germinal center cells. Short-term expansion of $CD33^+$ cells was observed within 2 weeks of culture (Fig. 2A, upper panel). $CD10^+$ cells started to appear at 2 weeks, and their numbers increased rapidly until 4 or 5 weeks of culture (Fig. 2A, middle panel). Cocultures on hMSC in the presence of SCF and FL led to generation of $1–5 \times 10^5$ $CD10^+$ cells from 2000 CB $CD34^+$ cells after 4 weeks of culture. Further addition of IL-7 to these cocultures had limited effect on $CD10^+$ cell production.

With regard to the transition into immature B cells, part of the generated $CD19^+$ cells began to express surface IgM after 4 weeks in coculture that included SCF and FL (Fig. 2A, lower panel; Fig. 2B). Surface phenotypes of the generated cells were analyzed after 6 weeks of culture (Fig. 2C), and most of the generated $CD10^+$ cells expressed CD19. Approximately 16.4% of $CD10^+$ cells expressed CD20. At that time, 32.5% of $CD10^+$ cells still showed surface expression of CD34. We did not detect any $CD3^+$ T-lineage cells or glycophorin A^+ erythroid cells.

Coculture of human $CD34^+$ cells on MS-5 stromal cells in the presence of SCF and G-CSF was reported to support human B lymphopoiesis [12]. Therefore, we compared B-lymphocyte production in hMSC coculture with added SCF plus FL vs SCF plus G-CSF. As shown in Figure 2D, the cocultures with SCF and FL generated B lymphocytes more efficiently than cocultures with SCF and G-CSF.

Therefore, we concluded that the coculture of CB $CD34^+$ cells on hMSC in the presence of SCF and FL was a suitable system for analyzing human B-lymphocyte development.

Screening for positive and negative regulators of human B lymphopoiesis

Because our coculture system was composed only of human-derived materials, except for FCS, we thought that it might be a suitable system for screening regulators of human B lymphopoiesis, and added several different low molecular weight inhibitors to the cocultures. Reactive oxygen species generation is related to induction of apoptosis in hematopoietic stem cells [33]. We confirmed that 100 μM N-acetylcysteine reduced apoptosis in murine hematopoietic stem cells by inhibiting reactive oxygen species generation. DUP697 is an inhibitor of Cox-2, which induces PGE_2 production. Endogenous PGE_2 was found in the supernatants of the cocultures and addition of 0.1 μM DUP697 blocked its production ($[PGE_2] = 0.74 \pm 0.40 \times 10^{-9}$ M without DUP697 and undetectable with DUP697). However, these inhibitors had no effect on human B-lymphocyte production (Fig. 3A). BIO is an inhibitor of a glycogen synthase kinase-3, which induces degradation of β-catenin [34]. Addition of BIO diminished the emergence of $CD10^+$ cells by approximately 30% (Fig. 3A), indicating that signals mediated by β-catenin inhibit human B-lymphocyte development. SB431542 is an inhibitor of ALK4/5/7, which are receptors for the TGF-β superfamily. Addition of SB431542 enhanced expansion of $CD10^+$ cells in a dose-dependent manner (Figs. 3A and B). Importantly, the influence of SB431542 on B-lymphocyte progenitors was greater than its influence on myeloid progenitors, because the percentage of $CD10^+$ cells increased significantly in the cocultures with added SB431542 (Fig. 3C).

Therefore, we determined that β-catenin and the TGF-β superfamily members act as negative regulators of human B-lymphocyte development in our coculture system.

Activin A and TGF-β1 negatively regulate human B lymphopoiesis

Among members of the TGF-β superfamily, TGF-β1 recognizes ALK-1 and -5, activin A binds to ALK-4, and BMP-4 uses ALK-2, -3, and -6 as receptors [24]. We added recombinant proteins as well as neutralizing Abs of these molecules to the cocultures. Production of human B lymphocytes decreased in a dose-dependent manner with the addition of TGF-β1, but not with activin A or BMP-4 (Fig. 4). A neutralizing Ab for activin A enhanced B-lymphocyte production approximately threefold, but neutralizing Abs for TGF-β1 and BMP4 had no effect (Fig. 5A). The physiological antagonist of activin A, follistatin, enhanced human B-lymphocyte production in a dose-dependent manner (Fig. 5B), and the percentage of $CD10^+$ cells in the generated cells increased markedly with the addition of follistatin (Fig. 5C), indicating that activin A downregulates human B lymphopoiesis more efficiently than myelopoiesis.

Therefore, both activin A and TGF-β1 inhibit human B-lymphocyte development, while BMP-4 has no apparent regulatory effect on human B-lymphocyte progenitor cells in our cocultures.

Both activin A and TGF-β1 inhibit early onset of human B lymphopoiesis

We next examined the effect of SB431542 on the frequency of B-lymphocyte progenitor cells. In limiting dilution culture with SB431542, the frequency of progenitor cells capable of generating $CD10^+$ cells increased significantly (Fig. 6A). Moreover, when a neutralizing Ab for activin A was added to cocultures derived from subpopulations of CB $CD34^+$ cells, the production of B lymphocytes, but not myeloid cells, was significantly enhanced (Fig. 6B and C). The enhancing effect on B-lymphocyte production was particularly great when the cocultures were started from $CD34^+CD38^-$ and $CD34^+CD38^+CD10^-$ subpopulations.

Therefore, both activin A and TGF-β1 inhibit early onset of human B lymphopoiesis.

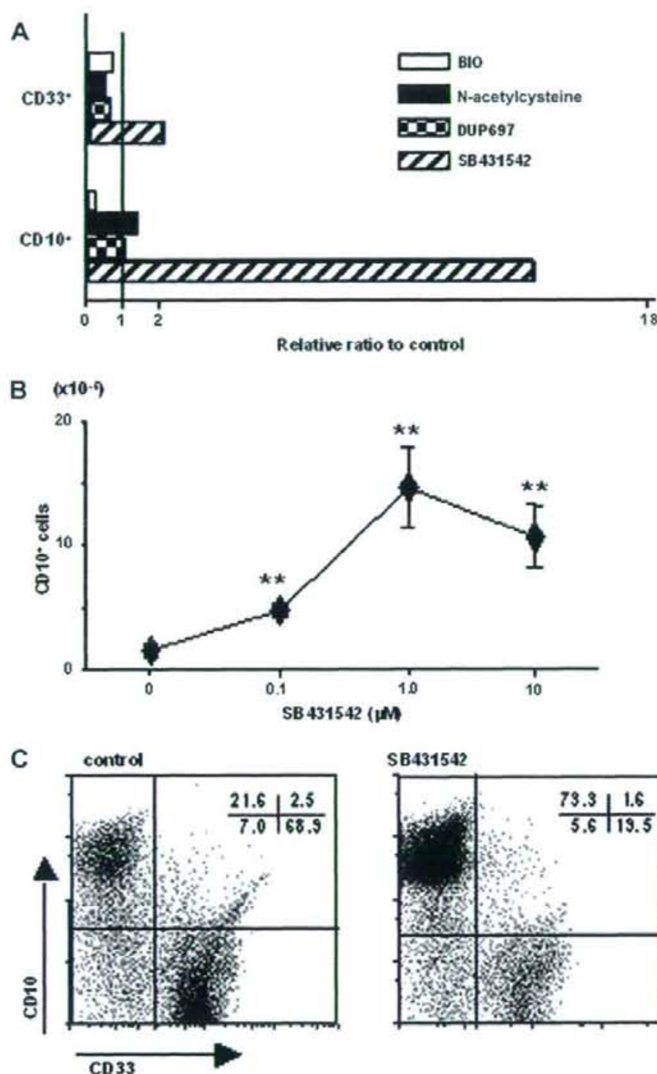


Figure 3. Addition of an inhibitor for activin receptor-like kinase [ALK]-4/5/7 to the cocultures selectively enhances human B-lymphocyte production. (**A**) 5 nM BIO (glycogen synthase kinase-3 inhibitor), 100 μM N-acetylcysteine (reactive oxygen species inhibitor), 0.1 μM DUP697 (Cox-2 inhibitor) or 10 μM SB431542 (ALK-4/5/7 inhibitor) was added to the cocultures of CB CD34$^+$ cells (2000 cells/well) on human mesenchymal stem cells (hMSC) in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL). At day 28, the generated CD33$^+$ or CD10$^+$ cells were calculated from the recovered total cell numbers and the percentages of the positive cells confirmed by flow cytometry. Data are shown as mean in duplicated samples. Similar results were obtained in three independent experiments. (**B**) The indicated concentrations of SB431542 were added to the cocultures of CB CD34$^+$ cells (2000 cells/well) on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL. Data are shown as mean ± standard deviation of numbers of the generated CD10$^+$ cells in triplicated samples. Statistically differences from control values (without SB431542) are shown with two ($p < 0.01$) asterisks. Similar results were obtained in two independent experiments. (**C**) Purified CB CD34$^+$ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL with or without 1.0 μM SB431542. At day 28, the generated cells were stained with phycoerythrin (PE)-CD10 and fluorescein isothiocyanate (FITC)-CD33, and analyzed with flow cytometry. Similar results were obtained in four independent experiments.

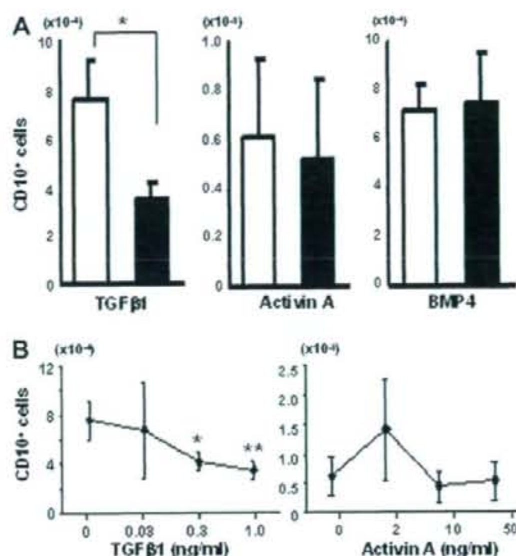


Figure 4. Transforming growth factor (TGF)-β1 negatively regulates human B lymphopoiesis in the cocultures. (**A**, **B**) Purified cord blood (CB) CD34$^+$ cells (2000 cells/well) were cultured on human mesenchymal stem cells (hMSC) in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL) with control (open column), 1 ng/mL TGF-β1, 50 ng/mL activin A, or 50 ng/mL bone morphogenetic protein (BMP)-4 (closed column) (**A**), with the indicated concentrations of TGF-β1 or activin A (**B**). At day 28, the generated CD10$^+$ cells were calculated from total cell numbers and percentages of the positive cells confirmed by flow cytometry. Data are shown as mean ± standard deviation in triplicated samples. Statistically differences from control values are shown with one ($p < 0.05$) or two ($p < 0.01$) asterisks. Similar results were obtained in three independent experiments.

Effect of TGF-β superfamily members on the transition into immature B cells

As shown in Figure 2, part of CD19$^+$ cells develop into IgM$^+$ immature B cells after 4 weeks in culture. Therefore, we evaluated the effects of TGF-β superfamily members on the transition into immature B cells in coculture. Blocking ALK-4/5/7 by SB431542 failed to influence the percentage of the IgM$^+$ population in the cultured CD19$^+$ cells at 5 weeks of culture (Fig. 7A). Similarly, we did not detect any difference in the percentage of IgM$^+$ cells by the addition of recombinant TGF-β1, a neutralizing Ab for activin A, or follistatin (Fig. 7B, C, and D).

Therefore, we did not observe any influence by TGF-β superfamily members on the transition into immature B cells in our human B-lymphocyte coculture system.

Coculture production of activin A and TGF-β1

Using reverse transcription PCR, we confirmed that RNAs of activin A type I and type II receptors were expressed by both CB CD34$^+$ cells and CD34$^-$ cells (Fig. 8A). hMSC expressed RNAs of the TGF-β superfamily members (Fig. 8B). Supernatants from the cocultures contained 1700 ± 410 pg/mL activin A and 40.8 ± 19.4 pg/mL TGF-β1 at day 3 of culture, and 3200 ± 130 pg/mL activin A and 114.7 ± 16.1 pg/mL TGF-β1 at day 10 (Fig. 8C). When we examined BM sections from normal healthy individuals, we detected activin A- and TGF-β1–positive cells (data not shown).

Therefore, both activin A and TGF-β1 are produced in our cocultures and in human BM.

Discussion

We established a novel coculture system to analyze human B lymphopoiesis. In our system, hMSC could support the commitment and differentiation of CB CD34$^+$ cells into CD10$^+$ cells, followed by transition into IgM$^+$ immature B cells. hMSC retained their capacity for cell-to-cell contact inhibition; therefore, we could continue the cocultures for up to 6 weeks without passage of hMSC. Moreover, our coculture system is a biologically relevant model for human B-lymphocyte development in that it excludes the effects of xenograft materials. When IL-7 was added to the SCF and FL-containing cocultures, only a few IgM$^+$ cells appeared. Thus, adding the combination of SCF and FL enhanced the B-lymphocyte–supporting capacity of hMSC.

We found that the addition of SB431542, an inhibitor for ALK-4/5/7, enhanced the output of CD10$^+$ cells markedly. Follistatin, a physiological inhibitor of activin A, and a neutralizing Ab for activin A enhanced B-lymphocyte production, while a neutralizing Ab for TGF-β1 had no discernable effect. TGF-β1, but not activin A, suppressed B-lymphocyte production in a dose-dependent manner. The different effects of inhibitors and factors on human B-lymphocyte production seemed to be related to the fact that the culture supernatant contained a much higher concentration of activin A than of TGF-β1. Adding a neutralizing Ab for BMP-4 or recombinant BMP-4 protein itself had no effect on B-lymphocyte production. Therefore, the strength of the ability to suppress human B lymphopoiesis seemed to be activin A > TGF-β1 > BMP-4.

Members of the TGF-β superfamily are implicated in control of many biological processes, such as cell cycle, cell growth and differentiation, and lymphocyte development and function [25]. The importance of these regulatory cytokines on immune homeostasis is reflected by the phenotypes of TGF-β1–deficient mice that develop autoimmune diseases with production of autoantibodies [35], although suppression of self-reactive lymphocyte clones involves actions of TGF-β1 on both B and T lymphocytes [36]. There are several reports suggesting that the TGF-β superfamily can modulate B-lymphocyte proliferation, expression of surface antigen receptors, and Ab secretion [37–40]. In murine B lymphopoiesis, TGF-β [7] and activin

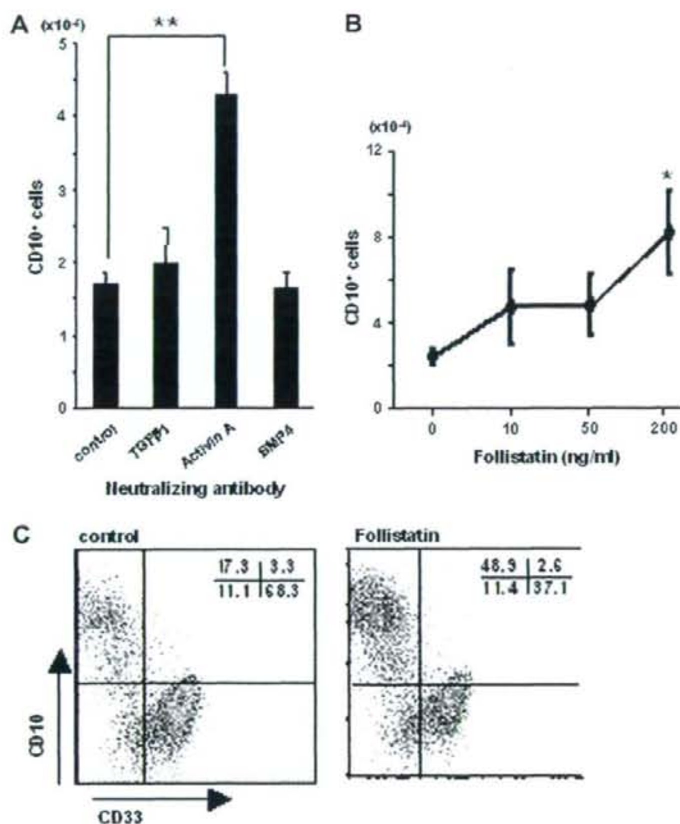


Figure 5. Inhibition of activin A enhances human B lymphopoiesis in the cocultures. (**A**, **B**) Purified cord blood (CB) $CD34^+$ cells (2000 cells/well) were cultured on human mesenchymal stem cells (hMSC) in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL) with 10 μg/mL antibodies (Abs) for TGF-β1, activin A, or bone morphogenetic protein (BMP)-4 (**A**), or with the indicated concentrations of follistatin (**B**). At day 28, the generated $CD10^+$ cells were calculated from total cell numbers and percentages of the positive cells confirmed by flow cytometry. Data are shown as mean ± standard deviation in triplicated samples. Statistically differences from control values are shown with one ($p < 0.05$) or two ($p < 0.01$) asterisks. Similar results were obtained in three independent experiments. (**C**) Purified CB $CD34^+$ cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL with or without 200 ng/mL follistatin for 4 weeks. The generated cells were stained with phycoerythrin (PE)-CD10 and fluorescein isothiocyanate-CD33, and analyzed with flow cytometry. Similar results were obtained in three independent experiments.

A [26,41] inhibit early onset of B-lymphocyte development. Because production of $CD10^+$ cells from CB $CD34^+$ cells was suppressed by both activin A and TGF-β1, our results from the coculture experiments coincide well with previous reports about the negative regulatory effects of these molecules on murine B-lymphocyte development. Notably, production of $CD10^+$ cells was influenced by the inhibition of TGF-β superfamily members even when the $CD34^+CD38^-$ stem cell population was used to initiate cocultures, and the influence was greater in the cocultures derived from $CD34^+CD38^-$ and $CD34^+CD38^+CD10^-$ cells than in cocultures derived from $CD34^+CD38^+CD10^+$ cells. These data suggest that the TGF-β superfamily inhibits early onset of human B lymphopoiesis. Furthermore, production of $CD10^+$ cells was also influenced when the inhibitor was added after 2 weeks of coculture (data not shown), indicating that the TGF-β superfamily might suppress the proliferation of relatively differentiated B-lymphocyte progenitors. Thus, members of the TGF-β superfamily are likely to suppress human B lymphopoiesis at a wide range of differentiation stages. This hypothesis is supported in part by our reverse transcription PCR data showing that both $CD34^+$ cells and $CD34^-$ cells express receptors for the TGF-β superfamily. Although a number of investigators have reported regulatory effects of the TGF-β superfamily on class switching

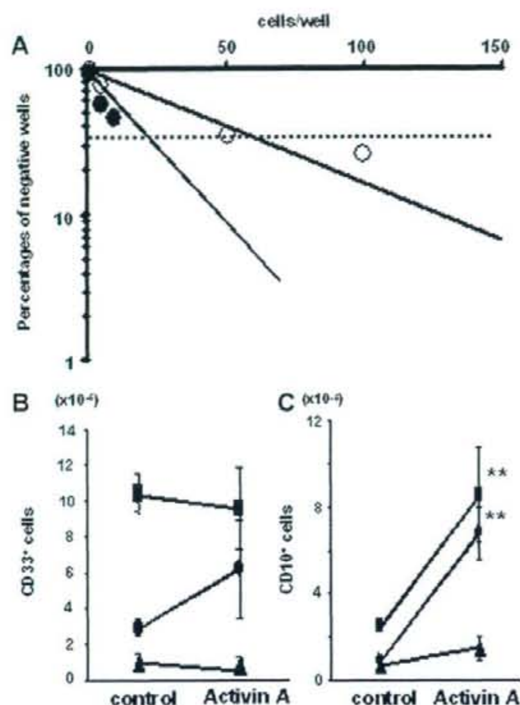


Figure 6. Transforming growth factor (TGF)-β superfamily inhibit early onset of human B lymphopoiesis. (A) Limiting dilution assays were performed in 96-well plates. Cord blood (CB) $CD34^+$ cells were cultured on human mesenchymal stem cells (hMSC) at indicated concentrations in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL) with (closed circle) or without 10 μM SB431542 (open circle). After 28 days of coculture, wells with cell expansion were scored. The expanded cells were analyzed by flow cytometry and calculated the well number of cultures generated $CD10^+$ cells. Similar results were obtained in three independent experiments. (B, C) Purified CB $CD34^+$ cells were stained with allophycocyanin (APC)-CD34, fluorescein isothiocyanate (FITC)-CD38, and phycoerythrin (PE)-CD10. $CD38^-$ cells (square), $CD38^+$ $CD10^-$ cells (circle), and $CD38^+$ $CD10^+$ cells (triangle) were then sorted with FACSAria. The sorted cells (2000 cells/well) were cultured on hMSC in the presence of 10 ng/mL SCF and 5 ng/mL FL with or without 10 μg/mL antibodies for activin A for 4 weeks. Numbers of the generated $CD33^+$ cells (B) or $CD10^+$ cells (C) were evaluated. Data are shown as mean ± standard deviation in triplicated samples. Statistically differences from control values are shown with two ($p < 0.01$) asterisks. Similar results were obtained in two independent experiments.

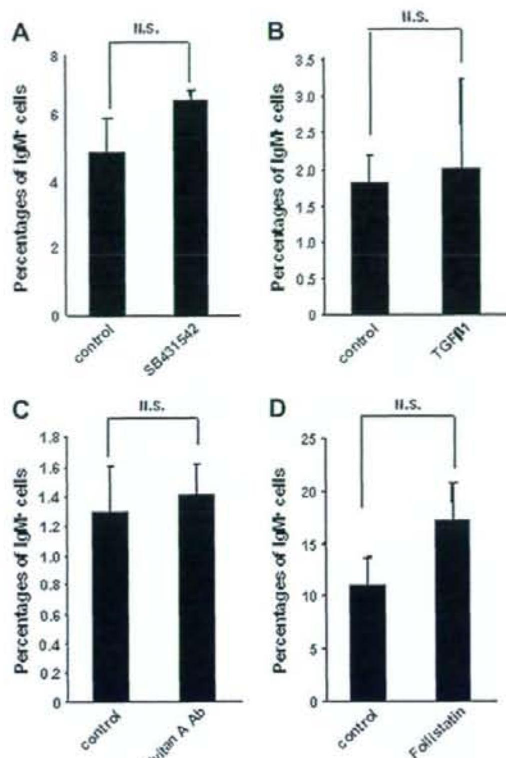
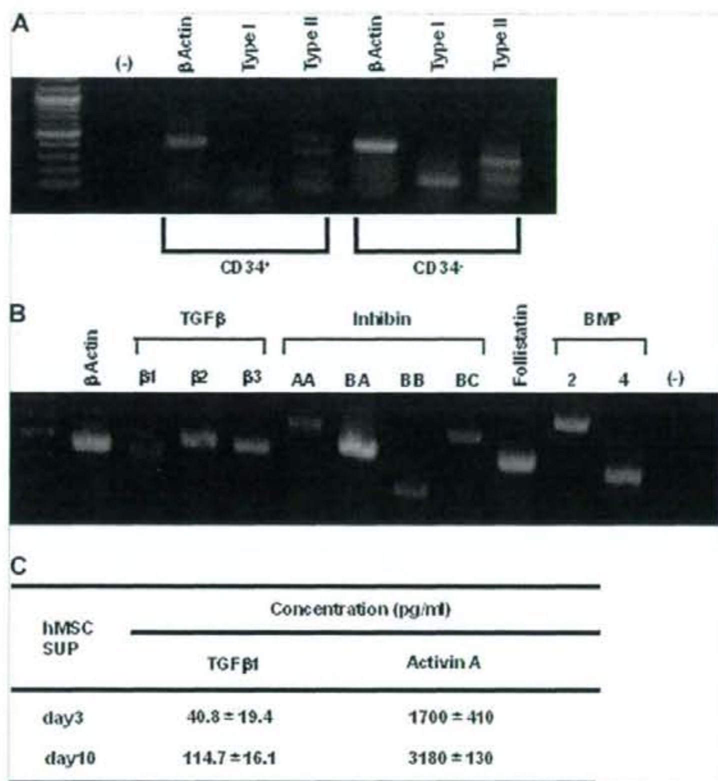


Figure 7. Transition from pre-B cells into immature B cells is not influenced by manipulating the transforming growth factor (TGF)-β superfamily in the cocultures. (A–D) Purified cord blood (CB) $CD34^+$ cells (2000 cells/well) were cultured on human mesenchymal stem cells (hMSC) in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL) with 1 μM SB431542 (A), with 0.3 ng/mL TGF-β1 (B), with 10 μg/mL an anti-activin A (C), or with 200 ng/mL follistatin (D). At day 42, the generated cells were stained with fluorescein isothiocyanate (FITC)-IgM and phycoerythrin (PE)-CD19, and analyzed with flow cytometry. Similar results were obtained in two independent experiments. NS = not significant.

and immunoglobulin secretion, little information about the influence on the transition from pre-B to immature B cells is available. We found that the percentage of surface IgM^+ cells in $CD19^+$ cells was not influenced by manipulating the TGF-β superfamily, while the production of $CD19^+$ cells was affected. Therefore, the transition into immature B cells is unlikely to be influenced by the TGF-β superfamily.

In conclusion, our coculture system of CB $CD34^+$ cells on hMSC in the presence of SCF and FL is suitable for analyzing the regulatory mechanisms of human B-lymphocyte development. With this system, we showed that members of TGF-β superfamily, activin A and TGF-β1, are negative regulators of human B-lymphocyte development at a range of differentiation stages. We expect that our coculture system will be applicable to a variety of research and development processes, such as screening for regulatory molecules or drugs that influence human B-lymphocyte development, evaluating B-lymphocyte progenitors in patients with B-cell malignancies, and cloning human B-lymphocyte–supportive molecules.



hMSC SUP	Concentration (pg/ml)	
	TGFβ1	Activin A
day3	40.8 ± 19.4	1700 ± 410
day10	114.7 ± 16.1	3180 ± 130

Figure 8. Production of activin A and transforming growth factor-β1. (**A**, **B**) Expression of RNAs of type I and type II receptor of activin A in cord blood (CB) CD34$^+$ and CD34$^-$ cells (**A**) and of the various TGF-β superfamily members in human mesenchymal stem cells (hMSC) (**B**) was analyzed with reverse transcription polymerase chain reaction. (**C**) Purified CB CD34$^+$ cells were cultured on hMSC in the presence of 10 ng/mL stem cell factor (SCF) and 5 ng/mL Flt3-ligand (FL). Culture supernatant was collected just before medium change at day 3 and day 10, and subjected to enzyme-linked immunosorbent assay for activin A or TGF-β1, respectively. Data are shown as mean ± standard deviation (n = 3 each).

Acknowledgments

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Diffuse large B-cell lymphoma showing an interfollicular pattern of proliferation: a study of the Osaka Lymphoma Study Group

A Yamauchi, J Ikeda, I Nakamichi, M Kohara, S Fukuhara,[1] M Hino,[2] Y Kanakura,[3] H Ogawa,[4] H Sugiyama,[5] A Kanamaru[6] & K Aozasa

Department of Pathology, Osaka University Graduate School of Medicine, [1]*First Department of Internal Medicine, Kansai Medical University,* [2]*Department of Clinical Haematology and Diagnostics, Osaka City University, Graduate School of Medicine,* [3]*Department of Haematology and Oncology, Osaka University Graduate School of Medicine, Osaka,* [4]*Department of Internal Medicine, Hyogo College of Medicine, Hyogo,* [5]*Department of Functional Diagnostic Science, Osaka University Graduate School of Medicine, and* [6]*Department of Haematology, Kinki University School of Medicine, Osaka, Japan*

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Diffuse large B-cell lymphoma showing an interfollicular pattern of proliferation: a study of the Osaka Lymphoma Study Group

Aims: Diffuse large B-cell lymphoma (DLBCL) usually proliferates effacing lymph follicles. In occasional cases, tumour cells show an interfollicular pattern of proliferation preserving lymph follicles. The aim was to analyse clinicopathological findings in DLBCL showing an interfollicular pattern of proliferation to determine whether this type of lymphoma is a distinct entity of DLBCL.

Methods and results: Clinicopathological findings in 12 cases of DLBCL showing an interfollicular pattern of proliferation [interfollicular group (IF)] were examined and compared with those in 30 cases of DLBCL with ordinary morphology [control group (CG)]. IF showed a significantly lower lactate dehydrogenase level and International Prognostic Index scores than CG ($P = 0.023$ and $P < 0.01$, respectively). The frequency of localized disease, clinical stage 1 and 2, in IF was higher than that in CG ($P = 0.016$). A morphologically polymorphous pattern of proliferation was found in seven of 12 cases (58.3%) in IF, which was higher than that in CG, five (16.7%) of 30 cases ($P < 0.01$). Clonality analysis with the polymerase chain reaction method revealed that all 11 IF cases examined showed a monoclonal pattern. Immunohistochemically, the majority (11 of 12) of IF cases showed a non-germinal centre B-cell phenotype and the frequency was higher than that in CG ($P = 0.021$).

Conclusion: Diffuse large B-cell lymphoma with an interfollicular pattern of proliferation shows distinct clinical and pathological findings from ordinary DLBCL.

Keywords: diffuse large B-cell lymphoma, immunophenotype, interfollicular pattern, prognosis

Abbreviations: CG, control group; DLBCL, diffuse large B-cell lymphoma; EBER, Epstein–Barr virus-encoded RNA; EBV, Epstein–Barr virus; GCB, germinal centre B-cell type; HPF, high-power field; IF, interfollicular group; IPI, International Prognostic Index; JH, joining region of immunoglobulin heavy chain gene; LDH, lactate dehydrogenase; MALT, mucosa-associated lymphoid tissue; NHL, non-Hodgkin's lymphoma; OLSG, Osaka Lymphoma Study Group; PCR, polymerase chain reaction; PTCL-NOS, peripheral T-cell lymphoma, not otherwise specified; WHO, World Health Organization

Address for correspondence: Amane Yamauchi, MD, Department of Pathology (C3), Osaka University Graduate, School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. e-mail: yamauchi@molpath.med.osaka-u.ac.jp

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Introduction

Diffuse large B-cell lymphoma (DLBCL), the most common type of malignant lymphoma in the World Health Organization (WHO) classification, is defined as diffuse proliferations of large neoplastic mature B cells.[1] When based on this criterion, DLBCL comprises morphologically, immunohistochemically and clinically heterogeneous tumours rather than one single entity. Generally, tumour cells in DLBCL show a diffuse pattern of proliferation totally effacing the nodal architecture. In the WHO classification, several morphological variants of DLBCL are listed: centroblastic, immunoblastic, T cell/histiocyte rich, anaplastic, plasmablastic, and DLBCL with expression of full-length anaplastic lymphoma receptor tyrosine kinase (ALK).[1] In occasional cases, however, tumour cells show an interfollicular pattern of proliferation preserving lymph follicles. This type of lymphoma has not been analysed separately from other types of DLBCL.

Several attempts have been made to classify DLBCL into biologically and clinically relevant subgroups. One of these approaches defined the three groups of DLBCL which display gene expression profiles similar to normal B-cell counterparts: the germinal centre B-cell type (GCB), activated B-cell type (ABC), and type 3 DLBCL.[2,3] Approximately 50% of adult DLBCLs are reported to be of the GCB subtype.[2]

Diffuse large B-cell lymphoma usually shows diffuse proliferation replacing or destroying lymph follicles. There is a possibility that DLBCL with an interfollicular pattern of proliferation preserving lymph follicles is derived from some peculiar subset of B cells other than GCB cells and may represent a distinct subtype of DLBCL. In the present study, clinicopathological findings in DLBCL showing an interfollicular pattern of proliferation were analysed to determine whether this type of lymphoma is a distinct entity of DLBCL.

Materials and methods

PATIENTS

From November 1999 to April 2007, 3056 cases of lymphoproliferative disease were registered with the Osaka Lymphoma Study Group (OLSG), Japan. Histological samples in all cases were fixed in 10% formalin and routinely processed for paraffin embedding. Histological sections, cut at 4 μm, were stained with haematoxylin and eosin and an immunoperoxidase procedure (ABC method). All cases were reviewed by a panel of pathologists in the OLSG as a first step, and all suspected cases of malignant lymphoma were then immunophenotyped. As a result, 2498 cases were diagnosed as malignant lymphoma, of which 2271 (90.9%) were non-Hodgkin's lymphoma (NHL) and 227 (9.1%) were Hodgkin's lymphoma. There were 1097 cases of DLBCL, which constituted 48.3% of NHL. Twelve cases (1.1%) of DLBCL arose in lymph nodes and showed the interfollicular pattern of proliferation; clinicopathological and immunohistochemical findings in these cases [interfollicular group (IF)] were analysed. As a control group, 30 patients with DLBCL who were admitted to Osaka University Hospital during the period August 2000 to February 2006 were selected [control group (CG)] (Table 1). Because all of the IF cases were nodal in origin, we selected the ordinary DLBCL of nodal origin with adequate follow-up data. The range and median ages in the CG and IF groups were 42–87 years (median 63.2) and 42–82 years (median 68.2), respectively (Table 1).

Adequate clinical information was available in all patients. On the basis of the records of physical examination, surgical notes and pathological examination of the specimens, the Ann Arbor staging system was applied in all IF cases and 30 CG cases. The International Prognostic Index (IPI) score was calculated with five adverse factors [age > 60 years, Ann Arbor stage III and IV, Eastern Cooperative Oncology Group performance score 2–4, elevation of serum lactate dehydrogenase (LDH), and two or more extranodal lesions] present at the time of diagnosis.[4] For cases <60 years old, an age-adjusted IPI score was applied, in which advanced stage, low performance score and elevation of LDH were considered as adverse factors.[4] All patients received anthracycline-based chemotherapy, mostly a regimen including doxorubicin, cyclophosphamide, vincristine and prednisone. Rituxumab was given in seven of 12 IF cases and seven of 30 CG cases. Clinical outcome was evaluated according to the guidelines of the International Workshop to standardize response criteria for NHL.[5]

FOLLOW-UP

The IF patients were observed until 28 May 2007; the follow-up periods for survivors ranged from 4.3 to 65.6 months (median 20.3). Nine of 11 patients were alive at the end of the observation period. The 2-year actuarial survival rate was 100%.

IMMUNOHISTOCHEMISTRY

Monoclonal antibodies used were CD20, CD79a, CD3, CD5, CD8, CD43, CD138, Bcl-6, MUM-1, Bcl-2, Ki67 (DakoCytomation, Glostrup, Denmark), CD4 (NovoCastra, Newcastle, UK) and CD10 (Nichirei Biosciences,

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Table 1. Brief clinical findings

	Interfollicular group (n = 12)	Control group (n = 30)	P-value
Mean age (years)	68.2	63.2	
M:F	8:4	21:9	
Stage			
1 or 2	75% (9/12)	34% (10/29)	
1	5	3	
2	4	7	
3 or 4	25% (3/12)	66% (19/29)	
3	0	7	
4	3	12	0.016
Bulky mass			
No	92% (11/12)	90% (27/30)	
Yes	8% (1/12)	10% (3/30)	NS
LDH			
Normal	75% (9/12)	37% (11/30)	
High	25% (3/12)	63% (19/30)	0.023
IPI			
Low	55% (6/11)	11% (3/28)	<0.01
LI	27% (3/11)	39% (11/28)	
HI	9% (1/11)	39% (11/28)	
High	9% (1/11)	11% (3/28)	
B symptoms			
Absent	82% (9/11)	62% (11/16)	
Present	18% (2/11)	38% (6/16)	NS
BM involvement			
Positive	17% (2/12)	24% (7/29)	
Negative	83% (10/12)	76% (22/29)	NS

LI, Low intermediate; HI, high intermediate; BM, bone marrow; LDH, lactate dehydrogenase; IPI, International Prognostic Index.

Tokyo, Japan). Tonsils with reactive lymphoid hyperplasia served as external control tissues. We counted 1000 cells for Ki67 immunohistochemistry and calculated the Ki67 index as follows: number of Ki67+ cells/100 cells.

IN SITU HYBRIDIZATION

RNA *in situ* hybridization using the Epstein–Barr virus-encoded RNA (EBER)-1 probe was performed as previously described, with some modifications.[6] Briefly, 30-base oligonucleotide probes, 5′-AGACACCGTCCTC ACCACCCGGGACTTGTA-3′, which were the sense and antisense for a portion of the EBER-1 gene, a region of the Epstein–Barr virus (EBV) genome that is actively transcribed in latently infected cells, were synthesized using a DNA synthesizer. The Raji cell line was used as a positive control. As negative controls, the hybridizing mixture containing sense probe or antisense probe after RNase treatment was used.

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EVALUATION OF CLONALITY FOR B CELLS

DNA was extracted from the paraffin-embedded samples from the 11 IF cases as described previously.[7] Seminested polymerase chain reaction (PCR) was performed using primer pairs of frame-work (Fr)2A or Fr3A and LJH for round 1 and Fr2A or Fr3A and VLJH for round 2, as described previously.[8] In addition, the BIOMED-2 PCR protocol for Fr2 and Fr3 was applied as described previously.[9] The amplified products were electrophoresed in 6% polyacrylamide gel. DNA extracted from two CG cases and one B-cell lymphoma line (Raji) were examined in parallel. PCR mixture without DNA was examined as a negative control, and a case of chronic tonsillitis as a polyclonal control. Clonality analysis was not performed in one IF case because there was not enough paraffin-embedded material to extract DNA.

Table 2. Pathological features in interfollicular and control group of diffuse large B-cell lymphoma

	Interfollicular group (n = 12)	Control group (n = 30)	P-value
Proliferation pattern			
Monomorphous	5	25	<0.01
Polymorphous	7	5	
Fibrosis			
Present	5	13	NS
Absent	7	17	
Mitotic count (high-power field)			
Mean	4.3	3.6	NS
Range	0–10	0–10	

Results

CLINICAL FINDINGS

Clinical findings are summarized in Table 1. Sex ratio (M:F) and age (range and median) in the IF and CG were 2.0 and 42–82 years (median 68.2) and 2.3 and 42–87 years (median 63.2), respectively. Levels of LDH and IPI score at initial diagnosis were significantly lower in IF than in CG ($P < 0.23$ and $P < 0.01$, respectively). As for disease stage, frequency of localized disease, stage 1 and 2, was higher in IF (75%) than in CG (34%) ($P = 0.016$).

PATHOLOGICAL FINDINGS

At the site of interfollicular proliferation, there was a monomorphous pattern of proliferation of large lymphoid cells in five cases, and a polymorphous pattern containing small lymphocytes, plasma cells, eosinophils and macrophages together with large lymphoid cells in seven cases. The ratio of cases showing a polymorphous pattern in IF, seven (58.3%) of 12 cases, was higher than that in CG, five (16.7%) of 30 cases ($P < 0.01$) (Table 2). Varying degrees of fibrosis were relatively frequent in both groups. The mean mitotic count in IF [4.3/high-power field (HPF) (0.237 mm^2)] was higher than that in CG (3.6/HPF), but the difference was not statistically significant. The proliferating large B-lymphoid cells were CD20+/CD79a+/CD3−. Intermingling small lymphoid cells among the proliferating B cells in the polymorphous proliferation were predominantly T lymphocytes in all but one case (Figure 1). The intermingled T lymphocytes comprised mixed CD4+ and CD8+ cells in the five of seven cases showing polymorphous proliferation. In the remaining two cases, intermingling T cells were predominantly CD8+.

One of the patients had received a resection of the left submandibular mass: histological diagnosis was an extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) type. One year later, a left subclavicular lymph node swelling appeared and was biopsied: histological diagnosis was DLBCL of IF pattern. Clonality analysis using PCR did not reveal a common clonal band between the two lesions, therefore we considered DLBCL of IF pattern in this case was *de novo* lymphoma rather than relapse of MALT lymphoma. There were no multiple biopsies in the other 11 IF patients.

IMMUNOHISTOCHEMICAL ANALYSIS FOR GERMINAL CENTRE B-CELL (GCB) OR NON-GCB PHENOTYPE

Based on the expression of CD10, Bcl-6 and MUM-1, the present cases of DLBCL were further classified into GCB and non-GCB types according to the decision tree proposed by Hans *et al.*[10] In brief, cases showing CD10+ or cases with CD10−/Bcl-6+/MUM-1− were categorized as GCB and the others as non-GCB. No cases of showed show CD10 positivity, whereas 28.6% of CG cases were CD10+. Of IF cases, 33.3% were positive for Bcl-6 and 66.7% for MUM-1. Of CG cases, 66.7% were positive for Bcl-6 and 50.0% for MUM-1. As a result, the frequency of the cases with GCB phenotype in IF, one (8.3%) of 12 cases, was significantly lower than that in CG, 12 (42.9%) of 28 cases

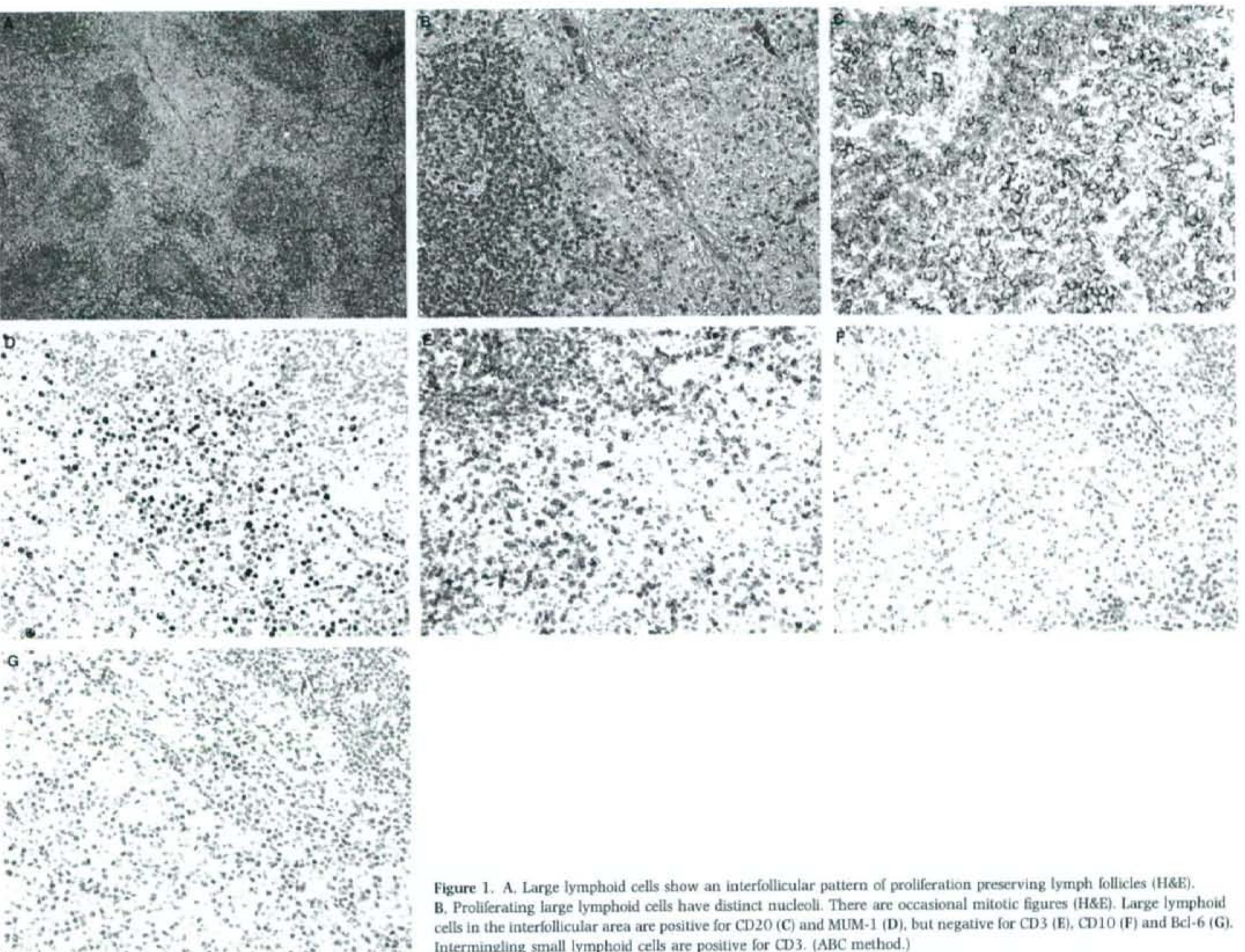

Figure 1. A, Large lymphoid cells show an interfollicular pattern of proliferation preserving lymph follicles (H&E). B, Proliferating large lymphoid cells have distinct nucleoli. There are occasional mitotic figures (H&E). Large lymphoid cells in the interfollicular area are positive for CD20 (**C**) and MUM-1 (**D**), but negative for CD3 (**E**), CD10 (**F**) and Bcl-6 (**G**). Intermingling small lymphoid cells are positive for CD3. (ABC method.)

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Table 3. Immunohistochemical findings in interfolicular and control group of diffuse large B-cell lymphoma

Antibody	Interfollicular group	Control group	*P*-value
CD10, no (%)	0/12 (0)	8/28 (29)	0.011
Bcl-6, no (%)	4/12 (33)	18/27 (67)	NS
MUM-1, no (%)	8/12 (67)	13/26 (50)	NS
GCB type, no (%)	1/12 (8)	12/28 (43)	0.021
Non-GCB type, no (%)	11/12 (92)	16/28 (57)	0.021
CD5, no (%)	0/11 (0)	0/14 (0)	NS
CD43, no (%)	3/12 (25)	1/22 (5)	NS
CD138, no (%)	2/12 (17)	0/18 (0)	0.049
Bcl-2, no (%)	6/12 (50)	12/23 (52)	NS
Ki67, mean (range)	64 (20–90)	66 (30–90)	NS

GCB, germinal centre B-cell type.

($P = 0.021$) (Table 3). There were no difference in Ki67 index between IF and CG (Table 3).

IN SITU HYBRIDIZATION

None of the 11 IF cases or of the 15 CG cases were EBV+.

CLONALITY ANALYSIS

With use of the Fr3A primer, six IF cases showed monoclonal and the remaining five oligoclonal bands. With use of the Fr2A primer, six cases showed monoclonal and the remaining five no bands. With use of Fr2 primer of BIOMED-2 protocol, five cases showed monoclonal, two oligoclonal and the other four no bands. With use of the Fr3 primer of BIOMED-2 protocol, seven cases showed monoclonal, one oligoclonal, one smear (polyclonal) and the other two no bands. As a result, every case showed a monoclonal pattern of proliferation with at least one primer. Three control samples, two ordinary DLBCL cases and one B-cell line, showed a monoclonal pattern. Polyclonal smear was found in the sample from tonsillitis and no band in the negative control (Figure 2).

PROGNOSIS

The overall 2-year survival rate was 100.0% in IF and 90.2% in CG. Two of the IF patients died of pneumonia 38 and 6 months after the start of treatment.

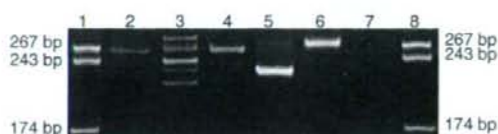

Figure 2. Clonality analysis with use of Fr2A primer. Lanes 1 and 8, size marker; lanes 2–4, cases of diffuse large B-cell lymphoma (DLBCL) with interfollicular pattern of proliferation; lane 5, B-cell lymphoma line (Raji); lane 6, case of DLBCL with ordinary morphology; lane 7, negative control (without DNA). Monoclonal band was found in lanes 2, 4, 5 and 6, and oligoclonal bands in lane 3.

Discussion

Diffuse large B-cell lymphoma usually shows a diffuse pattern of proliferation effacing the nodal architecture, including lymph follicles. On the other hand, tumour cells in peripheral T-cell lymphomas, not otherwise specified (PTCL-NOS) basically proliferate in the paracortical region frequently preserving lymph follicles. DLBCL of IF therefore resembles PTCL-NOS as a pattern of proliferation. In addition, two-thirds of the IF cases showed a polymorphous pattern of proliferation containing normal inflammatory cells, further mimicking the histological picture of PTCL-NOS. DLBCL of IF showed a relatively favourable prognosis, whereas PTCL-NOS usually shows an unfavourable course. Therefore, distinction of these two conditions is essential for making decisions on treatment modalities. Definite distinction of DLBCL showing interfollicular proliferation from the PTCL-NOS could be made only after immunohistochemical evaluation.

Among cases with DLBCL, clinical findings in IF cases were different from those in CG cases. IF showed significantly lower LDH levels and IPI scores than CG. As for disease stage, the frequency of localized disease, stage 1 and 2, was higher in IF than in CG. As a result, IF showed more favourable overall survival than CG, although not statistically significantly so, possibly due to the small number of cases and short follow-up period evaluated in this study.

A polymorphous pattern of proliferation containing large B cells, small lymphocytes, plasma cells and macrophages was common in IF, which contrasts with the monomorphous pattern exclusively containing large B cells found in CG. Such a polymorphous pattern is occasionally found in the lymphoproliferative condition developed in immunocompromised hosts in association with EBV;[11–13] however, none of the present cases was EBV+.

The relatively favourable prognosis, low IPI score and predominantly polymorphous pattern of prolifera-

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tion observed in IF argued against the neoplastic nature of this group. The clonality of the proliferating B cells in IF was then evaluated. The analysis revealed the monoclonal nature of the proliferating B cells in all 11 cases examined, justifying the diagnosis of DLBCL. Because EBV genome was not detected in the current cases, a polymorphous pattern of growth might suggest the following possibilities: concomitant reactive T lymphocytes as a host reaction to the proliferating large B-lymphoid cells, production of cytokines by the proliferating B cells which induce an inflammatory reaction,[14] and an early stage of DLBCL preserving the lymphoid follicles originally present in the lymph node.

Immunohistochemistry revealed that most (11 of 12) of the IF cases showed the non-GCB phenotype, and this frequency was significantly higher than that in CG ($P = 0.021$). No IF cases showed positivity for germinal centre marker CD10. Colomo *et al.*[15] have classified DLBCL into four groups according to the expression pattern of four antigens, CD10, Bcl-6, MUM-1 and CD138. According to their classification, tumour cells in seven of 11 IF cases expressed antigens identical to postgerminal centre stage, CD10–/Bcl-6(+or–)/MUM-1+/CD138– or CD10–/Bcl-6–/MUM-1+/CD138+. One case showed a germinal centre phenotype, CD10–/Bcl-6+/MUM-1–/CD138–. The differentiation stage of tumour cells in the remaining three cases was not defined, i.e. CD10–/Bcl-6–/MUM-1–/CD138–.

Marafioti *et al.*[16] have described the morphological and immunohistochemical characteristics of interfollicular large B cells in normal lymph nodes: these cells possessed dendritic processes. They speculated that interfollicular large B cells may be post-GCB cells and a precursor of activated B-cell-like DLBCL. They postulated that interfollicular large B cells might represent the cell of origin of some DLBCLs, especially those which tend preferentially to involve the interfollicular T-cell area in lymph nodes. Although dendritic processes were not found in the tumour cells in the current IF cases, immunophenotypical characteristics in the current cases (CD10–, Bcl-6– and MUM-1+), were similar to those in the interfollicular large B cells described by Marafioti *et al.* These findings suggest that DLBCL showing an interfollicular pattern of proliferation might be a neoplasm of interfollicular large B cells.

In conclusion, DLBCL with an interfollicular pattern of proliferation shows clinical and pathological findings distinct from ordinary DLBCL. Follow-up study of a large number of cases and precise immunophenotypical and genetic analyses are necessary.

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Arteriosclerosis obliterans associated with anti-cardiolipin antibody/β2-glycoprotein I antibodies as a strong risk factor for ischaemic heart disease in patients with systemic lupus erythematosus

J. Nojima[1], Y. Masuda[1], Y. Iwatani[2], H. Kuratsune[3], Y. Watanabe[4], E. Suehisa[1], T. Takano[1], Y. Hidaka[1] and Y. Kanakura[5]

Objective. The main objective of this study was to clarify the role of aPLs in the pathogenesis of arteriosclerosis obliterans (ASO), ischaemic heart disease (IHD) and cerebral vascular disorder (CVD) in patients with SLE.

Methods. We evaluated 155 patients with SLE by using objective tests for diagnosing ASO, IHD and CVD and laboratory tests including ELISA for aCL/β2-glycoprotein I antibodies (aCL/β2-GPI) and anti-phosphatidylserine/prothrombin antibodies (anti-PS/PT).

Results. Twenty-five (16.1%) of the 155 SLE patients were diagnosed with ASO. Both aCL/β2-GPI and anti-PS/PT levels were significantly higher in SLE patients with ASO (mean ± S.E., 104.3 ± 38.8 U/ml for aCL/β2-GPI, $P < 0.01$; 72.6 ± 48.9 U/ml for anti-PS/PT, $P < 0.05$) than in SLE patients without ASO (22.8 ± 9.9 U/ml for aCL/β2-GPI; 18.3 ± 4.4 U/ml for anti-PS/PT). Multivariate logistic analysis including aCL/β2-GPI, anti-PS/PT and traditional risk factors (hypercholesterolaemia, hypertension and diabetes mellitus) confirmed that the presence of aCL/β2-GPI was the most significant risk factor for ASO in SLE patients [odds ratio (OR) 3.45; 95% CI 1.40, 8.56; $P < 0.01$]. Furthermore, the prevalence of ASO was associated strongly with IHD (OR 11.8; 95% CI 3.45, 40.1; $P < 0.0001$) but not CVD (OR 1.84; 95% CI 0.65, 5.21; $P = 0.25$).

Conclusions. The presence of aCL/β2-GPI contributes to the risk of development of ASO, which may represent an important mechanism for the pathogenesis of IHD in patients with SLE.

Key words: Systemic lupus erythematosus, Anti-phospholipid antibodies, Arteriosclerosis obliterans, Ischaemic heart disease, Cerebral vascular disorder.

Introduction

The aPLs are a distinct group of auto-antibodies that occur in a variety of autoimmune diseases, particularly SLE [1, 2]. It is now generally accepted that aPLs do not bind primarily to the negatively charged phospholipid itself, but rather to complexes of the phospholipid and phospholipid-binding proteins [3–6]. The most common and best characterized aPLs are aCL/β2-glycoprotein I antibodies (aCL/β2-GPI), anti-phosphatidylserine/prothrombin antibodies (anti-PS/PT) and lupus anticoagulant (LA) activity [7, 8]. aCL/β2-GPI and anti-PS/PT are detected with solid-phase immunoassays, typically ELISAs [2, 7, 9, 10]. LA activity is the activity of aPLs that inhibit certain *in vitro* phospholipid-dependent coagulation reactions [11].

Several clinical studies have established that the presence of aCL/β2-GPI, anti-PS/PT and/or LA activity is associated with clinical events such as arterial and/or venous thromboembolic complications and obstetric complications [12–14]. APS is diagnosed both by clinical findings (recurrent arterial and/or venous thrombosis and obstetric complications) and laboratory evidence of persistent aPLs [15]. Although arterial and/or venous thrombosis and recurrent fetal loss are the major manifestations in APS patients, the spectrum of clinical manifestations associated with aPLs has broadened recently [16]. The nervous system in patients with aPLs is frequently affected, with migraines, balance disorders, stroke and atypical multiple sclerosis being prominent. Other features such as thrombocytopenia, psychiatric manifestations, livedo reticularis, haemolytic anaemia and cardiac valve abnormalities are also related to the presence of aPLs. Recently, atherosclerosis, ischaemic heart disease (IHD) and cerebral vascular disorder (CVD) were described as very important clinical manifestations associated with aPLs [17].

Atherosclerosis obliterans (ASO), IHD and CVD are major causes of mortality in patients with SLE [18, 19]. In ASO, large peripheral arteries are obstructed due to atherosclerosis. ASO has been hypothesized to be the major cause of IHD and/or CVD [17]. Many studies have suggested that SLE patients have increased prevalence of accelerated atherosclerosis [17, 19, 20]. Atherosclerosis occurs due to traditional risk factors such as hypercholesterolaemia, hypertension, diabetes mellitus, adiposity and smoking [17, 19, 21–23]; however, these traditional risk factors alone do not explain the high prevalence of atherosclerosis in patients with SLE. The association between SLE and atherosclerosis may be attributed to additional risk factors related closely to inflammation and autoimmunity [24]. In particular, several autoantibodies and their respective autoantigens may be possible factors in the development and progression of the atherosclerotic process in SLE [24].

Recently, a number of studies suggested that aPLs bind to the phospholipid/plasma protein complex on endothelial cells and/or the monocyte surface and that these antibodies increase levels of tissue factor (TF) messenger RNA (mRNA) synthesis and TF expression on monocytes and vascular endothelial cells [16, 25]. The increased TF activity on these cells in response to aPLs may be a mechanism for development of atherosclerosis in SLE patients with aPLs [16, 25].

In the present study, we evaluated 155 SLE patients using objective tests for diagnosis of ASO, IHD and CVD and by using laboratory tests, including aCL/β2-GPI-ELISA and anti-PS/PT-ELISA, to investigate the role of aCL/β2-GPI

[1]Laboratory for Clinical Investigation, Osaka University Hospital, Suita, Osaka, [2]Division of Biomedical Informatics, Course of Health Science, Osaka University Graduate School of Medicine, Suita, [3]Department of Health Science, Faculty of Health Science for Welfare, Kansai University of Welfare Science, Kashihara, [4]Department of Physiology, Osaka City University Graduate School of Medicine, Abeno-ku and [5]Department of Hematology and Oncology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan.

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Correspondence to: J. Nojima, Laboratory for Clinical Investigation, Osaka University Hospital, 2-15 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: nojima@hp-lab.med.osaka-u.ac.jp

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and anti-PS/PT in the pathogenesis of ASO, IHD and CVD in patients with SLE.

Materials and methods

Patients

We studied 155 patients (144 females, 11 males; age range 8–82 yrs; mean 44.2 yrs) with SLE diagnosed according to the revised criteria of the American Rheumatism Association. These patients were evaluated with objective tests for diagnosis of ASO, IHD and CVD and laboratory tests, including aCL/β2-GPI-ELISA and anti-PS/PT-ELISA. As the first step for diagnosis of ASO, the 155 patients with SLE were evaluated by determination of the ankle brachial pressure index (ABI) and Fontaine Stages (I–IV). Twenty-five (16.1%) patients satisfied both of the following diagnostic criteria: moderate-to-severe attenuation of ABI (<0.9) and stable intermittent claudication compatible with Fontaine Stage II. We performed angiography in these 25 patients and confirmed that all 25 patients had occlusive disease of the artery that disturbed blood flow (ASO-positive group). The remaining 130 patients, who had no abnormality in the ABI test, a lower limb Doppler ultrasound and clinical symptoms of ASO, were classified as the ASO-negative group. Diagnosis of IHD and CVD was based on clinical manifestations and electrocardiography (ECG), coronary angiography (CAG), Doppler ultrasonography CT and/or MRI findings. Among all SLE patients, we confirmed that 15 (9.6%) patients had IHD (13 cases of angina pectoris and two cases of myocardial infarction) and 25 patients (16.1%) had CVD (23 cases of cerebral infarction and two cases of transient cerebral ischaemic attack). We also studied 150 control plasma samples from normal healthy volunteers. These samples had been previously taken from the staff of Osaka University Hospital (62 females, 88 males; age range 21–58 yrs; mean 40.8 yrs). None of them had any history of thrombotic complications, and there were no abnormalities found by blood testing (blood cell counts, coagulation tests, liver function tests and examinations for autoimmune activity). Blood samples were taken into vacuum tubes (5.0-ml total volume, Sekisui, Japan) containing 0.5 ml of 3.13% trisodium citrate ($Na_3C_6H_5O_7{\cdot}2H_2O$), and platelet-poor plasma was prepared by double centrifugation at 2800*g* for 15 min at 15°C. The plasma samples were frozen at −80°C until batch assays could be performed. Written informed consent was obtained from all study participants. This research was approved by the Institutional Review Board of Osaka University Hospital.

Detection of aCL/β2-GPI

Concentrations of aCL/β2-GPI were measured with an aCL/β2-GPI ELISA Kit (Yamasa Shoyus Co., Ltd, Japan). The cardiolipin-coated wells were washed three times with 250 μl of PBS (pH 7.4) containing 0.05% Tween-20 (PBS–Tween). The wells were then incubated with 50 μl of purified human β2-GPI (30 μg/ml) (β2-GP I^+ wells) or with 50 μl of PBS containing 0.5% BSA (0.5% BSA–PBS) (β2-GP I^- wells) for 30 min at room temperature. After incubation, 50 μl of plasma sample (diluted 101 times with 0.5% BSA–PBS) was added to both β2-GP I^+ wells and β2-GP I^- wells. To exclude the effect of β2-GPI present in plasma, we diluted plasma samples 101 times with PBS. Measurement of aCL/β2-GPI was not influenced by the β2-GPI in plasma because the final concentration of β2-GPI was <1 μg/ml. A 50 μl volume of calibration standard sera was added to β2-GP I^+ wells. Following a 60-min incubation at room temperature, the wells were washed three times with PBS–Tween and then incubated with 100 μl of peroxidase-labelled anti-human IgG for 30 min at room temperature. Wells were then washed three times with PBS–Tween and 100 μl of 0.3 mM tetramethylbenzidine solution containing 0.003% of H_2O_2 was added to each well. After a 30-min incubation at room temperature, the reaction was terminated by addition of 100 μl of 2N H_2SO_4, and the absorbance was measured at 450 nm. The absorbance levels for each sample in the β2-GP I^+ and β2-GP I^- wells were compared for the evaluation of β2-GPI dependency of antibody binding. In this experiment, all samples in β2-GP I^+ wells showed higher levels of absorbance than those in β2-GP I^- wells. The levels of aCL/β2-GPI were derived from the calibration curve from β2-GP I^+ wells.

Detection of anti-PS/PT

Concentrations of anti-PS/PT were measured with an anti-PS/PT IgG ELISA Kit (Cosmic Corporation, Japan). This kit detects PS-dependent anti-PT antibodies (aPS/PT) in human plasma. The PS-coated wells were washed three times with 300 μl of PBS containing 0.5% BSA, 5 mM $CaCl_2$ and 0.05% Tween-20 (Wash buffer). The wells were then incubated with 50 μl of purified human PT (30 μg/ml, PT^+ wells) or with 50 μl of 0.5% BSA–PBS (PT^- wells) for 60 min at room temperature. PT was added to wells coated with PS to refine the PS–PT complex. After washing, 100 μl of plasma sample (diluted 101 times with 0.5% BSA–PBS containing 5 mM $CaCl_2$) was added to both PT^+ and PT^- wells. Calibration standard sera (100 μl) were added to PT^+ wells and incubated for 60 min at room temperature. The second incubation allows any anti-PS/PT present to bind to the immobilized PS–PT complex. After washing, 100 μl of peroxidase-labelled anti-human IgG was added to both PT^+ and PT^- wells and incubated for 60 min at room temperature. A third incubation allows the enzyme label to bind to anti-PS/PT that has attached to the wells. A 100 μl of 0.3 mM tetramethylbenzidine solution containing 0.003% of H_2O_2 was added to each well after washing and then incubated for 30 min at room temperature. The reaction was stopped by addition of stop solution, and absorbance of the resulting yellow colour product was measured spectrophotometrically at 450 nm. Levels of anti-PS/PT were calculated from the calibration curve obtained with PT^+ wells.

Detection of LA activity

LA activity was detected with both the diluted Russell Viper Venom Time (dRVVT; Gradipore Ltd, Sydney, Australia) and STACLOT LA (Diagnostica Stago) tests. The dRVVT and STACLOT LA tests were performed with commercially available screening and confirmatory tests.

Statistical analysis

The non-parametric Mann–Whitney test and the Kruskal–Wallis test were used to compare levels of aCL/β2-GPI and anti-PS/PT between ASO patients, non-ASO patients and control subjects. As an approximation of the relative risk, the odds ratio (OR) and 95% CI were calculated for several putative risk factors with multivariate logistic regression analysis with the statistical program Stat Flex (version 4.2, Artech, Inc., Osaka, Japan). An OR was considered to be statistically significant when the lower limit of the 95% CI was >1.0. In the multivariate logistic regression analysis, $P < 0.05$ was considered statistically significant.

Results

Relation between LA activity and aCL/β2-GPI and/or anti-PS/PT

We studied levels of aCL/β2-GPI and anti-PS/PT in 150 normal healthy control subjects. The levels, detected by ELISA, were log transformed with the Stat Flex program to approximate normality before statistical analysis was performed. The mean + 3 S.D. in the 150 normal controls were chosen as the cut-off point for each antibody level. Cut-off values for aCL/β2-GPI and anti-PS/PT

were 3.5 and 10.2 U/ml, respectively. For the SLE patients, the result was regarded as positive when the absorbance exceeded each cut-off value. The aCL/β2-GPI and anti-PS/PT were detected in 61 (39.4%) and 42 (27.1%) of the 155 SLE patients, respectively. Thirty-four patients had both aCL/β2-GPI and anti-PS/PT, 27 had aCL/β2-GPI alone and 8 had anti-PS/PT alone.

It was recently reported that the presence of LA activity is the strongest risk factor for thromboembolic events in patients with SLE [12]. However, LA activity is heterogeneous with respect to the specificities and functional capacities of the antibodies, which recognize different antigens, including complexes of phospholipid/plasma proteins such as CL/β2-GPI and PS/PT [2, 26, 27]. More recently, some studies showed anti-PS/PT and aCL/β2-GPI to be independently responsible for LA activity [7, 8]. Therefore, we examined the relation between LA activity and aCL/β2-GPI and/or anti-PS/PT in our 155 SLE patients. LA activity was present in 56 (36.1%) of the 155 SLE patients. Forty-four (72.1%) of 61 aCL/β2-GPI-positive cases had LA activity and 37 (88.1%) of 42 anti-PS/PT-positive cases had LA activity. Multivariate logistic regression analysis revealed that the prevalence of LA activity correlated strongly with the presence of aCL/β2-GPI and anti-PS/PT (OR 9.2 and 19.4, respectively; 95% CI 3.52, 23.8 and 6.18, 60.9, respectively; $P < 0.001$ and $P < 0.001$, respectively).

Relation between the presence of aCL/β2-GPI and/or anti-PS/PT and the prevalence of ASO

We evaluated 155 patients with SLE using objective tests for diagnosis of ASO. Twenty-five patients were assigned to the ASO-positive group. The remaining 130 patients with SLE, who showed no abnormalities on the objective tests for diagnosis of ASO, were considered ASO-negative (ASO-negative group). Levels of aCL/β2-GPI and anti-PS/PT were compared between the ASO-positive group and ASO-negative group. As shown in Fig. 1, levels of both aCL/β2-GPI and anti-PS/PT were significantly higher in the ASO-positive group (aCL/β2-GPI, mean ± S.E., 104.3 ± 38.8 U/ml, $P < 0.001$; anti-PS/PT, 72.6 ± 48.9 U/ml, $P < 0.05$) than in the ASO-negative group (aCL/β2-GPI, 22.8 ± 9.9 U/ml; anti-PS/PT, 18.3 ± 4.4 U/ml).

The aCL/β2-GPI and anti-PS/PT were significantly present more in ASO-positive patients (aCL/β2-GPI, 16/25 cases, 64.0%, $P < 0.01$; anti-PS/PT, 10/25 cases, 40.0%, $P < 0.05$) than in ASO-negative patients (45/130 cases, 34.6%; 32/130 cases, 24.6%; respectively). The results of logistic regression analysis of risk factors for ASO are shown in Table 1. In this analysis, all values were analysed as positive or negative irrespective of the concentrations of antibodies. The logistic regression analysis of risk factors for ASO revealed that the presence of aCL/β2-GPI was a strong risk factor for ASO (OR 3.36; 95% CI 1.37, 8.20, $P < 0.01$). However, the presence of anti-PS/PT was not reliable as a risk factor for ASO in patients with SLE.

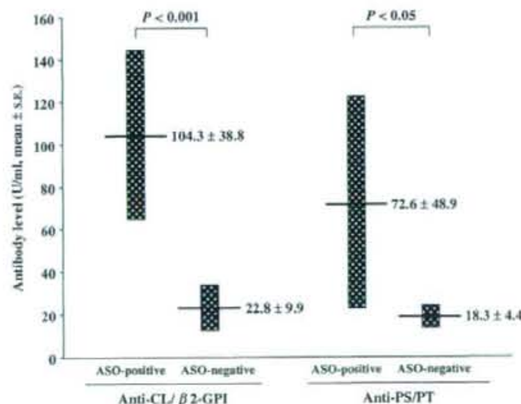

FIG. 1. Levels of aCL/β2-GPI and anti-PS/PT in 155 SLE patients with or without ASO.

Comparison between aCL/β2-GPI and traditional risk factors

Because traditional risk factors, including hyperlipaemia, hypertension and diabetes, are reported to contribute to the prevalence of ASO [17, 21], we examined the presence of these traditional risk factors in 155 SLE patients with or without ASO. Hyperlipaemia was diagnosed as LDL cholesterol levels of >140 mg/dl or as triglyceride levels of >150 mg/dl. Hypertension was defined by a systolic blood pressure of >140 mmHg and diastolic blood pressure of >90 mmHg. Diagnosis of diabetes mellitus was made according to the criteria of the Japanese Diabetes Society. Hyperlipaemia, hypertension and diabetes mellitus were detected in 44 (28.4%), 41 (26.5%) and 19 (12.3%) of the 155 SLE patients, respectively.

The results of multivariate logistic analysis, including aCL/β2-GPI and traditional risk factors (hyperlipaemia, hypertension and diabetes mellitus), are shown in Table 2. Hyperlipaemia, hypertension and diabetes mellitus were not reliable risk factors for ASO in patients with SLE (OR 1.09, 0.93 and 0.51, respectively; 95% CI 0.41, 2.92; 0.34, 2.56; and 0.11, 2.44, respectively; $P = 0.86$, $P = 0.89$, $P = 0.40$, respectively), and the presence of aCL/β2-GPI was the most significant risk factor for ASO in SLE patients (OR 3.45; 95% CI 1.40, 8.56; $P < 0.01$).

Relation between the presence of ASO and the prevalence of IHD and/or CVD

It has been suggested that atherosclerosis may contribute to development of IHD and CVD [17, 24]. Therefore, we examined the relation between the presence of ASO and the prevalence of IHD and/or CVD in our 155 SLE patients. The prevalence of IHD was significantly higher in SLE patients with ASO (9/25 cases, 36.0%, $P < 0.0001$, Table 3) than in those without ASO (6/130 cases, 4.6%). In contrast, there was no statistically significant difference in the prevalence of CVD between SLE patients with ASO (6/25 cases, 24.0%) and those without ASO

TABLE 1. Association between the presence of each aPL and the prevalence of ASO

	ASO		Logistic analysis		
Antibodies	Sensitivity (%)	Specificity (%)	OR	95% CI	P-value
Anti-CL/β2-GPI	64.0	65.4	3.36	1.37, 8.20	<0.01
Anti-PS/PT	40.0	75.4	2.04	0.84, 4.99	0.12

Statistical analyses consisted of univariate and multivariate logistic analyses. An OR was considered statistically significant when the lower limit of the 95% CI was >1.0. $P < 0.05$ was considered statistically significant to indicate risk.

TABLE 2. Multivariate logistic regression analysis of risk factors for ASO

	ASO		Logistic analysis		
Criterion variable	Sensitivity (%)	Specificity (%)	OR	95% CI	P-value
Anti-CL/β2-GPI	64.0	65.4	3.45	1.40, 8.56	<0.01
Hyperlipaemia	32.0	72.3	1.09	0.41, 2.92	0.86
Hypertension	28.0	73.8	0.93	0.34, 2.56	0.89
Diabetes	8.0	86.9	0.51	0.11, 2.44	0.40

Statistical analyses consisted of univariate and multivariate logistic analyses. An OR was considered statistically significant when the lower limit of the 95% CI was >1.0. $P < 0.05$ was considered statistically significant.

(19/130 cases, 14.6%). Multivariate logistic analysis revealed that the prevalence of ASO was strongly associated with IHD (OR 11.8; 95% CI 3.45, 40.1; $P < 0.0001$) but not CVD (OR 1.84; 95% CI 0.65, 5.21; $P = 0.25$; Table 3).

Relation between the prevalence of IHD and the presence of ASO, aCL/β2-GPI and/or anti-PS/PT

SLE patients were divided into the following four groups according to their complications (ASO and/or IHD): Group A, ASO-positive patients who had IHD ($n = 9$); Group B, ASO-positive patients who had no IHD ($n = 16$); Group C, ASO-negative patients who had IHD ($n = 6$); and Group D, ASO-negative patients who had no IHD ($n = 124$). Levels of aCL/β2-GPI and anti-PS/PT were compared among these four groups. As shown in Fig. 2, the aCL/β2-GPI level was significantly higher in Group A (mean ± S.E., 135.7 ± 67.9, $P < 0.001$) and Group B (86.7 ± 48.2, $P < 0.05$) than in Group D (23.8 ± 10.3), but no statistical difference was observed between Group A and Group B. Furthermore, the aCL/β2-GPI level in Group A was significantly higher than that in Group C (135.7 ± 67.9 *vs* 2.3 ± 0.8, $P < 0.01$). There was no statistical difference in the aCL/β2-GPI level between Group C and Group B.

The anti-PS/PT level was significantly higher in Group A (160.3 ± 134.6, $P < 0.05$) than in Group D (16.7 ± 4.3). However, there were no statistical differences in the levels of anti-PS/PT between Groups B (23.2 ± 9.9), C (52.1 ± 31.9) and D (16.7 ± 4.3).

TABLE 3. Association between the ASO and IHD and/or CVD

	ASO ($n = 25$ cases)	Non-ASO ($n = 130$ cases)	Logistic analysis		
			OR	95% CI	*P*-value
IHD (%)	9/25 (36.0)	6/130 (4.6)	11.8	3.45, 40.1	<0.0001
CVD (%)	6/25 (24.0)	19/130 (14.6)	1.84	0.65, 5.21	0.25

Statistical analyses consisted of multivariate logistic analyses. An OR was considered statistically significant when the lower limit of the 95% CI was >1.0. $P < 0.05$ was considered statistically significant.

Discussion

IHD such as angina pectoris and myocardial infarction are major causes of morbidity and mortality in patients with SLE [17]. However, the precise mechanism that underlies IHD in these patients remains unclear. In the present study, we showed that the presence of aCL/β2-GPI contributes to the risk of development of ASO, which may represent an important mechanism for the pathogenesis of IHD in patients with SLE.

Atherosclerosis is a pathological process that affects arterial walls [24]. Atherosclerosis is a chronic inflammatory response to the deposition of lipoproteins (cholesterol and triglycerides) in the walls of arteries [24]. In addition to the chronic inflammatory response, many components of the immune system, including monocytes and macrophages, T cells, autoantibodies and their respective autoantigens and cytokines secreted by cells within atherosclerotic plaques, are thought to be involved in the pathological processes that underlie development of atherosclerosis [24, 28].

It was previously reported that aPLs promote atherosclerosis by attracting monocytes to endothelial cells and/or promoting influx of oxidized low-density lipoprotein into macrophages [29]. Moreover, many studies have suggested that aPLs cause persistently high expression of TF on monocytes and vascular endothelial cells and that increased TF activity on these cells in response to aPLs may be involved in the pathogenesis of atherosclerosis in SLE patients [25, 30–36]. Furthermore, recent experimental evidence suggested that aPLs induce expression of *TF* gene and protein by monocytes and vascular endothelial cells by simultaneously and independently activating phosphorylation of p38 mitogen-activated protein kinase (MAP kinase), nuclear translocation and activation of NF-κB/Rel proteins, and phosphorylation of MEK-1/ERK proteins [16, 33, 34]. Activation of p38 MAPK increases activities of pro-inflammatory cytokines,

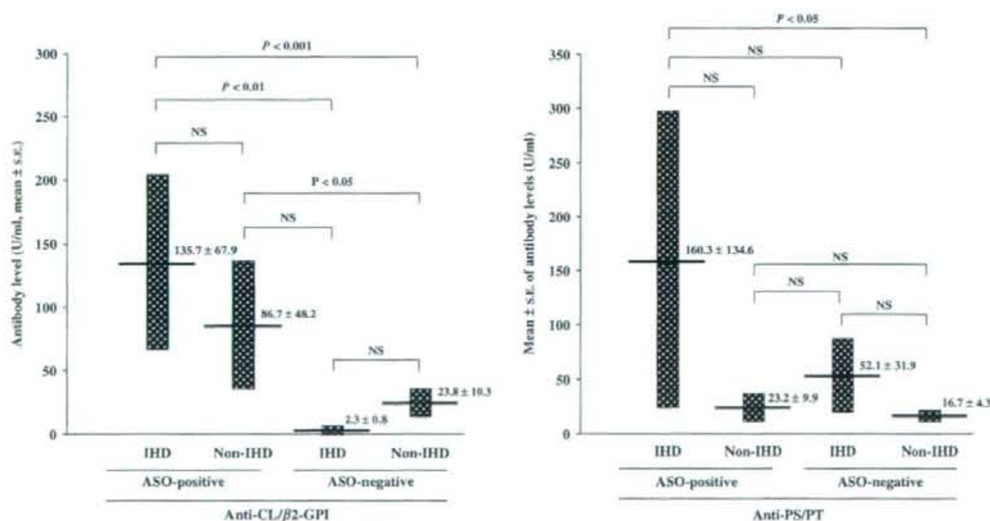

FIG. 2. Comparison of aCL/β2-GPI levels and anti-PS/PT levels among SLE patient groups. Group A, ASO-positive patients who had IHD ($n = 9$); Group B, ASO-positive patients who had no IHD ($n = 16$); Group C, ASO-negative patients who had IHD ($n = 6$); and Group D, ASO-negative patients who had no IHD ($n = 124$). NS: not significant.

such as TNF-α and IL-1β, and this may also contribute to the development of atherosclerosis in SLE patients with aPLs [24].

In the present study, we examined levels of aCL/β2-GPI and anti-PS/PT by using specific ELISAs in addition to LA activity detected by phospholipid-dependent coagulation assays in 155 SLE patients with or without ASO. The presence of LA activity is reported to be a strong risk factor for thromboembolic events in patients with SLE [12], and we confirmed that the presence of LA activity also contributed to the development of ASO in SLE patients (OR 2.67; 95% CI 1.12, 6.37). However, LA activity detected with a phospholipid-dependent coagulation assay can be heterogeneous, and some studies showed that expression of LA activity is dependent on the ability of aCL/β2-GPI and anti-PS/PT [7, 8]. Therefore, we investigated the role of aCL/β2-GPI and anti-PS/PT in the pathogenesis of ASO. It is important to note that antibodies detected by the anti-PS/PT ELISA differ from those detected by the aCL/β2-GPI ELISA and that anti-PS/PT and aCL/β2-GPI are independently responsible for LA activity. Although levels of both aCL/β2-GPI and anti-PS/PT were significantly higher in SLE patients with ASO than in SLE patients without ASO, logistic regression analysis of risk factors for ASO revealed that the presence of aCL/β2-GPI but not anti-PS/PT is a strong risk factor for ASO.

Traditional risk factors such as hyperlipaemia, hypertension and diabetes contribute to the prevalence of ASO [17, 19, 21–23]. Therefore, we examined the frequencies of these traditional risk factors in 155 SLE patients with or without ASO. Multivariate logistic analysis including aCL/β2-GPI and traditional risk factors (hyperlipaemia, hypertension and diabetes mellitus) confirmed that the presence of aCL/β2-GPI is the most significant risk factor for ASO in SLE patients. These results suggest that ASO in SLE patients may be mediated by the pro-inflammatory and pro-coagulant activities of aCL/β2-GPI and that the presence of aCL/β2-GPI promotes development and progression of ASO in patients with SLE.

Although the risk of IHD and/or CVD was reported to be increased in patients with SLE [17, 24], it is still not clear how aPL-associated ASO is related to this risk. Therefore, we examined the relation between the presence of ASO and the prevalence of IHC and/or CVD. Multivariate logistic analysis revealed that the presence of ASO is strongly associated with the prevalence of IHD in SLE patients. SLE patients with ASO had an 11.8 (OR) increased risk of IHD compared with that of SLE patients without ASO. Furthermore, we divided 155 SLE patients into four groups as described in the 'Materials and methods' section, and compared the levels of aCL/β2-GPI among these four groups. It is important to note that the aCL/β2-GPI level was significantly higher in SLE patients who had both ASO and IHD than in SLE patients who had IHD but not ASO. These results suggest that ASO caused by aCL/β2-GPI contributes to the risk of development of IHD in patients with SLE.

In contrast, the presence of ASO does not appear to be an important risk factor for the prevalence of CVD as indicated by the multivariate logistic regression analysis. In the present study, we confirmed that the prevalence of IHD was higher in the ASO group (36.0%, 9/25 patients) than in the non-ASO group (4.6%, 6/130 patients), whereas there was no statistically significant difference in the prevalence of CVD between the ASO group (24.0%, 6/25 patients) and the non-ASO group (14.6%, 19/130 patients). These results suggest that although the prevalence of IHD is strongly associated with the presence of ASO, the prevalence of CVD in SLE patients may be associated not only with ASO but also with other factors. With respect to the pathogenesis of CVD in SLE patients, platelet activation induced by aPLs is thought to be a possible mechanism of aPL-associated CVD [35, 37, 38]. Our previous studies indicated that SLE patients with both aCL/β2-GPI and anti-PS/PT have a high prevalence of cerebral infarction and that aCL/β2-GPI and anti-PS/PT promote platelet activation that may contribute to the pathogenesis of cerebral infarction in patients with SLE [39]. Because platelets in SLE patients are thought to be frequently activated by aPLs, infection, inflammation and endothelial cell damage, the high prevalence of CVD in SLE patients may be due not only to atherosclerosis but also to other factors such as platelet activation and endothelial cell damage.

In conclusion, in addition to the traditional risk factors for cardiovascular disease, the presence of ASO caused by aCL/β2-GPI is an important risk factor for development and progression of IHD in patients with SLE.

Rheumatology key messages

- This study shows that ASO caused by aCL/β2-GPI is a strong risk factor for IHD.
- This study contributes to the elucidation of the pathogenesis of IHD in SLE patients.

Disclosure statement: The authors have declared no conflicts of interest.

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