発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
松永恭子、矢野美由紀、浄慶幸江、増田由佳子、迫田裕之、炬口真理子、河野誠司、熊谷俊一、西郷勝康、杉本直志、永井謙一、高橋隆幸、矢部博樹、近藤信一、稲葉　亨、岡本茂高、古田香穂橋、メイラニ・シアンプルナワチ、巽　英二	顆粒発達の強い細胞を含むDR陰性AML（M2）：2例における Flt3ITD 検出と3例の塗抹標本形態	日本検査血液学会雑誌	7	264-269	2006
松永恭子、西郷勝康、橋本　誠、岸本麻奈美、炬口真理子、成田浩子、小澤利行、熊谷俊一	無侵襲ヘモグロビン測定装置の自己血貯血可否判断への応用と問題点	臨床病理	54	1106-1109	2006
Tsujimura S, Saito K, Kohno K, Tanaka Y.	Fragmented hyaluronan induces transcriptional up-regulation of the multidrug resistance-1 gene in CD4+ T cells.	J Biol Chem	281	38089-38097	2006
Fujii Y, Fujii K, Iwata S, Suzuki K, Azuma T, Saito K, Tanaka Y.	Abnormal intracellular distribution of NFAT1 in T lymphocytes from patients with systemic lupus erythematosus and characteristic clinical features.	Clin Immunol	119	297-306	2006
Nakano K, Saito K, Mine S, Matsushida S, Tanaka Y.	CD44 signaling up-regulates Fas Ligand expression on T cells leading to activation-induced cell death.	Apoptosis	12	45-54	2007
Yamanaka H, Tanaka Y, Sekiguchi N, Inoue E, Saito K, Kameda H, Iikuni N, Nawata M, Amano K, Shinozaki M, Takeuchi T.	Retrospective clinical study on the notable efficacy and related factors of infliximab therapy in a rheumatoid arthritis management group in Japan (RECONFIRM).	Mod Rheumatol	17	28-32	2007
Tsujimura S, Saito K, Nakayamada S, Tanaka Y.	Relevance of multidrug resistance 1 and P-glycoprotein to drug resistance in patients with systemic lupus erythematosus	Histol Histopathol	22	465-468	2007
Tokunaga M, Saito K, Kawabata D, Imura Y, Fujii T, Nakayamada S, Tsujimura S, Nawata M, Iwata S, Azuma T, Mimori T, Tanaka Y.	Efficacy of rituximab (anti-CD20) for refractory systemic lupus erythematosus involving the central nervous system	Ann Rheum Dis	in press		
Nakano K, Okada Y, Saito K, Tanikawa R, Sawamukai N, Sasaguri Y, Kohro T, Wada Y, Kodama M, Tanaka Y.	Rheumatoid synovial endothelial cells produce macrophage-colony stimulating factor leading to osteoclastogenesis in rheumatoid arthritis.	Rheumatology	in press		
Nakayamada S, Saito K, Nakano K, Tanaka Y.	β 1 integrin transduces an activation signal in T cells of patients with systemic lupus erythematosus.	Arthritis Rheum	in press		
齋藤和義、徳永美貴子、田中良哉	抗CD20抗体	最新医学	61 (5)	975-981	2006
齋藤和義、田中良哉	インフリキシマブ	整形・災害外科	49 (6)	667-675	2006

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
齋藤和義、田中良哉	膠原病に合併するニューモシスチス肺炎の早期診断、早期治療	リウマチ科	35 (1)	79-86	2006
名和田雅夫、齋藤和義、田中良哉	関節リウマチに対するインフリキシマブ使用の実際	リウマチ科	35 (5)	429-433	2006
名和田雅夫、齋藤和義、田中良哉	各種評価法を用いた最新治療評価の実際 —インフリキシマブ有効性評価を例として—	リウマチ科	36 (3)	281-286	2006
名和田雅夫、齋藤和義、田中良哉	MMP-3 によるインフリキシマブ有効性の予測	リウマチ科	36 (6)	600-604	2006
齋藤和義、田中良哉	膠原病に合併するニューモシスチス肺炎の早期診断、早期治療	分子リウマチ	4(1)	50-55	2007
Yoshida H, Endo H, Tanaka S, Ishikawa A, Kondo H, Nakamura T.	Recurrent paralytic ileus associated with strongyloidiasis in a patient with systemic lupus erythematosus.	Mod Rheumatol	16 (1)	44-47	2006
橋本　篤、松下礼子、飯塚進子、木村美保、高山陽子、田中住明、石川　章、遠藤平仁	メトトレキサート投与中の関節リウマチ患者に発症した悪性リンパ腫の 2 例	新薬と臨床	55 (12)	1896-1903	2006
吉田　秀、近藤啓文、遠藤平仁、西　正大、飯塚進子、木村美保、田中住明、石川　章	難治性虹彩炎を伴った Tattoo Sarcoidosis (刺青サルコイドーシス) の 1 例	臨床リウマチ	18 (3)	225-231	2006
原まさ子	全身性エリテマトーデス	Medical Practice	23 (4)	663-668	2006
原まさ子	ベリムマブ（抗 BlyS 抗体）と IDEC-131（抗 CD40L 抗体）	最新医学	61	969-974	2006
M.Kawamoto,M.Harigai, M.Hara,et al.	Epression and function of inducible co-stimulator in patients with systemic lupus erythematosus: possible involvement in excessive interferon-g and anti-double stranded DNA antibody production.	Arthritis Research & Therapy	8(3)		2006
Y.Kawaguchi, A.Tochimoto, M.Hara, et al.	Nos2 polymorphisms associated with the susceptibility to pulmonary arterial hypertension with systemic sclerosis: contribution to the transcriptional activity.	Arthritis Research & Therapy	8(3)	R104	2006
T.Sugiura, N.Maeno, M.Hara, et al.	A promotor haplotype of the onterleukin-18 gene is associated with juvenile idiopathic arthritis in the Japanese population.	Arthritis Research & Therapy	8	R60	2006
Y.Kawaguchi, E.Nishimagi, M.Hara, et al.	Intracellular IL-1a-binding proteins contribute to biological functions of endogenous IL-1a in systemic sclerosis fibroblasts.	PNAS	103 (39)	14501-14506	2006
S.Hirata, H.Okamoto, M.Hara,et al.	Dificient activity of von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura in the setting of adult-onset Still' s disease.	Rheumatology	45	1046-1047	2006
H.Kajiyama, H.Akama, H.Yamanaka, M.Hara, et al.	One third of Japanese patients with rheumatoid arthritis use complementary and alternative medicine.	Mod Rheumatol	16	355-356	2006
M.Iwatani, E.Inoue, T.Nakamura, M.Hara, et al.	Efficacy profile of bucillamine in rheumatoid arthritis patients in large observational cohort study, IORRA.	Mod Rheumatol	16	376-380	2006

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nakamura M, Tanaka Y, Satoh T, Kawai M, Hirakata M, Kaburaki J, Kawakami Y, Ikeda Y, Kuwana M.	Autoantibody to CD40 ligand in systemic lupus erythematosus: association with thrombocytopenia but not thromboembolism.	Rheumatology (Oxford)	45 (2)	150-156	2006
Kaneko Y, Suwa A, Ikeda Y, Hirakata M.	Pneumocystis jiroveci pneumonia associated with low-dose methotrexate treatment for rheumatoid arthritis: report of two cases and review of the literatures.	Mod. Rheumatol.	16 (1)	36-38	2006
Kimura T, Mukai M, Kaneko Y, Hirakata M, Okamoto S, Sakamoto M, Okada Y, Ikeda Y.	Unusual hemangioendothelioma of the liver with epithelioid morphology associated with marked eosinophilia: autopsy case.	Pathol. Int.	56 (11)	694-701	2006
Sato S, Kuwana M, Hirakata M.	Clinical Characteristics of Japanese patients with anti-OJ (anti-isoleucyl-tRNA synthetase) autoantibodies.	Rheumatology (Oxford)	in press		
Hirakata M, Suwa A, Takada T. Sato S, Nagai S, Genth E, Song YW, Mimori T, Targoff IN.	Clinical and immunogenetic features of patients with autoantibodies to asparaginyl-transfer RNA synthetase.	Arthritis Rheum.	in press		
平形道人	自己抗体陽性者に対する対処法および自己抗体の臨床的意義	内科	97 (4)	583-590	2006
平形道人	膠原病の肺病変（間質性肺炎）－活動性の評価、治療	Medical Practice	23 (4)	629-633	2006
平形道人	抗 Jo-1 抗体症候群	ドクターサロン	50 (5)	15-17	2006
平形道人	皮膚筋炎にみられる特徴的皮疹 / ヘリオトロープ疹・ゴットロン徴候	成人病と生活習慣病	36 (5)	473-475	2006
平形道人	抗 Jo-1 抗体およびその他の抗アミノアシル tRNA 合成酵素抗体	リウマチ科	36 (1)	32-38	2006
平形道人	膠原病診療のポイント：多発性筋炎 / 皮膚筋炎	診断と治療	94 (10)	1882 -1888	2006
平形道人	抗 Jo-1 抗体	日本医師会雑誌、「生涯教育リシーズ 70/ 最新臨床検査の ABC」	135 特別号 (2)	301	2006
平形道人	抗 Scl-70（DNA トポイソメラーゼ I）抗体	日本医師会雑誌、「生涯教育リシーズ 70/ 最新臨床検査の ABC」	135 特別号 (2)	302	2006
平形道人	抗 SS-A/Ro，抗 SS-B/La 抗体	日本医師会雑誌、「生涯教育リシーズ 70/ 最新臨床検査の ABC」	135 特別号 (2)	303	2006

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
平形道人	抗 LKM-1 抗体	日本医師会雑誌、「生涯教育リシーズ 70/ 最新臨床検査の ABC」	135 特別号 (2)	317	2006
花岡洋成、平形道人	膠原病における貧血	診断と治療	94 (11)	2063 -2067	2006
香月有美子、平形道人	その他の生物学的製剤：将来の低分子薬剤として期待される薬は？	Mebio	23 (12)	93-101	2006
Karassa FB, Afeltra A, Ambrozeic A, Chang D-M, Keyser FD, Doria A, Galeazzi M, Hirohata S, Hoffman IEA, Inanc M, Massardo L, Mathieu A, Mok CC, Morozzi G, Sanna G, Spindler AJ, Yzioufas AG, Yoshio T, Ioannidis JPA	Accuracy of anti-ribsosomal P protein antibody testing for the diagnosis of neuropsychiatric systemic lupus erythematosus. An international meta-analysis.	Arthritis Rheum	54	312-324	2006
Hirohata S	Role of bone marrow in the pathogenesis of rheumatoid arthritis.	Curr Rheum Rev	2	47-54	2006
Hirohata S, Miura Y, Tomita T, Yoshikawa H, Ochi T, Chiorazzi N	Enhanced expression of mRNA for nuclear factor kB1 (p50) in CD34+ cells of the bone marrow in rheumatoid arthritis.	Arthritis Res Ther	8	R54	2006
Hirohata S	Is the long-term use of systemic corticosteroids beneficial in the management of Behcet' s syndrome.	Nature Clin Practice Rheum	2	358-359	2006
広畑俊成	膠原病・セミナー / 膠原病の特異病変における診療のポイント、中枢神経病変	Medical Practice	23	624-638	2006
広畑俊成	特集 自己免疫疾患の新しい治療法－生物学的製剤を中心に－エファリズマブ（LFA 抗体）とナタリズマブ（VLA-4 抗体）	最新医学	61	993-999	2006
広畑俊成	特集 内科疾患最新の治療－専門家からのアドバイス　VI、膠原病・免疫・アレルギー Behcet 病	内科	97	1238-1239	2006
広畑俊成	関節リウマチ骨髄 CD34+ 細胞からの線維芽細胞様細胞の分化	Rheumatology Clinical Update	13	19-21	2006
広畑俊成	「関節リウマチ－積極的な治療へのパラダイム転換」Suggestion：関節リウマチの病態形成における骨髄異常について	治療学	40	732-733	2006
広畑俊成	特集　リウマチ・膠原病にみられる自己抗体－その対応抗原と臨床的意義　抗リボソーム P 抗体	リウマチ科	36	58-64	2006
広畑俊成	整形外科医が誤りやすい膠原病	臨床整形外科	41	962-969	2006

【V】研究成果の刊行物・別刷

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Fragmented Hyaluronan Induces Transcriptional Up-regulation of the Multidrug Resistance-1 Gene in CD4+ T Cells*

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P-glycoprotein, encoded by the multidrug resistance (*MDR*)-*1* gene, expels various drugs from cells resulting in multidrug resistance. We found previously that interleukin-2, a lymphocyte-activation cytokine, induces P-glycoprotein expression on lymphocytes. Lymphocyte activation involves adhesion with the extracellular matrix, such as hyaluronan, through adhesion molecules on lymphocytes. We investigated the transcriptional regulation of *MDR-1* in lymphocytes by fragmented hyaluronan. Fragmented hyaluronan (especially the 6.9-kDa form), not native high molecular hyaluronan, induced translocation of YB-1, a specific transcriptional factor for *MDR-1*, from the cytoplasm into the nucleus and resulted in the transcription of *MDR-1* and the expression of P-glycoprotein on lymphocytes in a dose-dependent manner. Transfection of YB-1 antisense oligonucleotides inhibited P-glycoprotein expression induced by fragmented hyaluronan. The fragmented hyaluronan induced significant P-glycoprotein expression on only activated CD4+ T cells, which highly expressed CD69, and resulted in excretion of intracellular dexamethasone added *in vitro*. Cyclosporin A, a competitive P-glycoprotein inhibitor, restored intracellular dexamethasone levels in CD4+ T cells. Anti-CD44 monoclonal antibody (Hermes-1) inhibited fragmented hyaluronan-induced YB-1 activation and P-glycoprotein expression in CD4+ T cells. We provide the first evidence that binding of fragmented hyaluronan to CD44 induces YB-1 activation followed by P-glycoprotein expression in accordance with activation of CD4+ T cells. Our findings imply that CD4+ T cell activation by fragmented hyaluronan, induced by characteristic extracellular matrix changes in inflammation, tumors, and other conditions, results in P-glycoprotein-mediated multidrug resistance.

The successful pharmacotherapy of various diseases, including tumors and autoimmune diseases, is often limited by multidrug resistance (1–3). Among the multiple mechanisms of multidrug resistance, overexpression of P-glycoprotein, a 170-kDa product of the multidrug resistance (*MDR*)[2]-*1* gene, has emerged as the major molecule involved in multidrug resistance during chemotherapy for various malignancies (1–6). Resistance to chemotherapy induced by P-glycoprotein is closely associated with the prognosis of human malignancies (1). P-glycoprotein is a member of the ATP-binding cassette transporter superfamily of genes and functions as an energy-dependent transmembrane efflux pump (7). Overexpression of P-glycoprotein results in a reduction of intracellular concentrations of xenobiotics, drugs, and poisons, such as vinca alkaloids, anthracyclines, verapamil, colchicines, antimalarials, and corticosteroids (2, 3, 8). P-glycoprotein is expressed on not only various types of tumor cells but also normal cells such as epithelial cells (9–11), CD34+ hematopoietic stem cells (12), and lymphocytes (13).

In various tumor cell lines, we and others have reported that transcription of *MDR-1* is directly regulated by the human Y box-binding protein-1 (YB-1), an *MDR-1* transcription factor, and that activation of YB-1 is induced in response to genotoxic stresses including multiple drugs (14, 15). In lymphocytes, our group reported previously that the transcription of *MDR-1* gene and expression of P-glycoprotein are induced by soluble factors including lymphocyte-activation cytokines such as interleukin (IL)-2, resulting in the activation of YB-1 and induction of its binding to DNA (13).

In addition to soluble factors, intercellular adhesion or adhesion to the extracellular matrix transduces signals via functional molecules on lymphocytes and these processes are involved in the regulation of immunological reactions (16, 17). Hyaluronan, a representative component of extracellular matrix, is a nonsulfated glycosaminoglycan bearing linear repeats of disaccharide-β-D-glucuronyl-β-D-*N*-acetylglucosamine (18).

The principal known receptor of hyaluronan is CD44 (19). CD44 is a 90-kDa transmembrane glycoprotein widely distributed on T lymphocytes, granulocytes, monocytes, fibroblasts, keratinocytes, and epithelial cells (20). Stimulation of CD44 with hyaluronan plays a role in various physiological functions, such as cell-cell adhesion, cell-substrate interactions, and lymphocyte recruitment, as well as pathological processes such as

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[2] The abbreviations used are: MDR, multidrug resistance; YB-1, Y box-binding protein 1; IL, interleukin; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorter; CMV, cytomegalovirus; TBS, Tris-buffered saline; RA, rheumatoid arthritis.

chronic inflammation and metastasis of malignant cells (19). Hyaluronan is usually generated as a high molecular weight polymer, a native hyaluronan (18), but a fragmented hyaluronan, a digested low molecular weight oligomer, is detected in certain pathological conditions, such as inflammation and tumors (21, 22). Several studies have reported that the biological activities of fragmented hyaluronan markedly differ from native hyaluronan in endothelial cell, various malignant cells, and macrophages (23–26).

Although *MDR-1* transcription in lymphocytes is induced by various activation stimuli such as cytokines, there is little information on whether stimulation with hyaluronan induces activation and *MDR-1* transcription in resting CD4+ T cells. Furthermore, it is also not clear at present whether the effect of fragmented hyaluronan on activation and regulation of MDR1 are different from native hyaluronan in CD4+ T cells. The present study was designed to determine the effects of fragmented hyaluronan on CD4+ T cell activation and its role in P-glycoprotein-mediated multidrug resistance, particularly in fragmented hyaluronan-rich microenvironments such as areas of chronic inflammation or tumors.

EXPERIMENTAL PROCEDURES

Isolation of Peripheral Blood Mononuclear Cells from Healthy Donors—We isolated peripheral blood mononuclear cells (PBMCs) from healthy donors by density gradient centrifugation using Lymphocyte Separation Medium 50494 (GE Healthcare) as described previously (27, 28). We confirmed that purified PBMCs contained more than 90% of lymphocytes (CD4, CD8, or CD20 positive cells) and less than 10% of CD14 positive monocytes by immunostaining. The study was approved by the Human Subject Research Committee of the University of Occupational and Environmental Health, School of Medicine, and informed consent was obtained from all donors who enrolled in the study.

CD4+ T Cells Isolation from PBMCs—CD4+ T cells were purified by negative selection using magnetic beads according to the recommended procedure supplied by the manufacturer (CD4 negative isolation kit; Dynal Biotech, Japan). The purity of the CD4+ T cell subset was determined by flow cytometry to be greater than 90%.

Reagents and mAbs—Fragmented (1.7, 6.9, and 40 kDa) and native (950 kDa) hyaluronan were kindly donated by the Tokyo Research Institute of Seikagaku (Tokyo, Japan). The following monoclonal antibodies (mAbs) were used as purified Ig in preparation of staining and analysis of cell surface or cytoplasmic molecules, blocking of CD44, and Western blotting analysis. MRK16 (a specific mAb against P-glycoprotein; Kyowa Medex, Tokyo), a specific antibody against YB-1 (a binding protein to the Y box and CCAAT box, which is critical for the cis-regulatory element that regulates drug-induced *MDR-1* gene expression (14)), CyChrome-conjugated CD4 mAb, CyChrome-conjugated CD8 mAb, phycoerythrin-conjugated CD69 mAb, fluorescein isothiocyanate-conjugated goat anti-mouse IgG Ab, fluorescein isothiocyanate-conjugated anti-rabbit IgG Ab (BD Biosciences), Hermes-1 (anti-human CD44 purified monoclonal antibody; Endogen), purified rat IgG_{2a} (anti-KLH, a control mAb for Hermes-1; BD Biosciences), and F4 (monoclonal anti-P-glycoprotein antibody; Sigma).

Flow Cytometric Analysis—Staining and flow cytometric analysis of PBMCs or CD4+ T cells were conducted by standard procedures as described previously using a FACScan (BD Biosciences) (27, 28). Briefly, PBMCs or CD4+ cells (2×10^5 cells/well) were initially incubated with polyclonal γ-globulin (10 μg/ml, Yoshitomi Pharmaceutical Co.) for blocking of Fc receptors and then incubated with MRK-16, followed by fluorescein isothiocyanate-conjugated anti-mouse IgG Ab in FACS medium consisting of phosphate-buffered saline, 0.5% human serum albumin, and 0.2% NaN_3 (Sigma). For the two-color analysis, we incubated PBMCs with CyChrome-conjugated CD4 mAb, CD8 mAb, or CD4+ cells with phycoerythrin-conjugated CD69 mAb after blocking of free anti-mouse IgG-binding sites with irrelevant antibodies. Monoclonal antibody two-color-stained cells were detected by electronic gating based on their CD4, CD8, or CD69 expression using a FACScan. Amplification of mAb binding was provided by a three-decade logarithmic amplifier. Quantification of the cell surface antigens on one cell was performed using QIFIKIT beads (Dako, Kyoto, Japan) as reported previously (27–29). The data were used to construct the calibration curve of mean fluorescence intensity *versus* antibody binding capacity. The cell specimen was analyzed on the FACScan and the antibody binding capacity calculated by interpolation on the calibration curve. When the green fluorescence laser detection was set at 500 nm in the FACScan, the antibody binding capacity was equal to (202.98 × exp (0.0092 × mean fluorescence intensity), ($R^2 = 0.9995$)). Subsequently, the specific antibody binding capacity was obtained after correcting for the background, and apparent antibody binding capacity of the negative control anti-mouse IgG Ab. The specific antibody binding capacity is the mean number of accessible antigenic sites per cell, referred to as antigen density and expressed in sites/cell.

Immunostaining and Confocal Microscopy Analysis—PBMCs or CD4+ T cells were plated onto a 12-well culture dish (2×10^5 cells/well) and incubated for 60 min at 37 °C in the presence or absence of 50 μg/ml 6.9-kDa fragmented hyaluronan in RPMI 1640 containing 5% fetal calf serum. The cells were then treated with 4% formaldehyde (Sigma) in FACS medium for 15 min and then with 0.1% saponin (Sigma) in FACS medium. The obtained cells were incubated with a specific antibody against YB-1 for 30 min at 4 °C. Subsequently, the cells were incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG Ab at saturating concentrations in FACS medium. We performed confocal analysis of YB-1 using a LSM 5-pascal inverted laser scan microscope (Carl Zeiss Microscope Systems, Germany).

Gel Shift Assay—Nuclear extracts from PBMCs were prepared as described previously (11) and then incubated with or without 50 μg/ml 6.9-kDa fragmented hyaluronan. In the next step, 4 μg of nuclear protein were preincubated for 20 min at room temperature in 15 μl of buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 4% glycerol, and 40 mM NaCl) containing 0.5 μg of poly(dI-dC) (GE Healthcare) and a ^{32}P-end-labeled double-stranded oligonucleotide containing the YB-1 consensus binding site (5′-GGGCAGTTTTAGCCA-

GCTCCTCCCTA-3′, 5′-GGGGTAGGGAGGAGCTGGCT-AAAACT-3′) as described previously (30). The reaction mixtures were electrophoresed on 4% polyacrylamide gels in 0.25× TAE buffer.

Transfection of Antisense Oligonucleotides of YB-1 in PBMCs—YB-1 antisense expression plasmid (PRC/CMV AS) was constructed as described previously (31). We transfected 2 μg of PRC/CMV AS or control vacant vector into 2×10^6 PBMCs in a 6-well culture dish using a cationic liposome-mediated transfection method, with cationic lipid reagents (DMRIE-CTM, Invitrogen) according to the instructions provided by the manufacturer (32–34). Forty-eight h after transfection, the cells were incubated for 6 h at 37 °C in the presence or absence of 5 μg/ml 6.9-kDa fragmented hyaluronan.

Reverse Transcription-Polymerase Chain Reaction—After 6 h of incubation with or without 50 μg/ml of 6.9-kDa fragmented hyaluronan in RPMI 1640 (Nissui, Tokyo) containing 5% fetal calf serum (Bio-Pro, Karlsruhe, Germany), total cellular RNA from CD4+ T cells was isolated by a single step isolation procedure with ISOGEN (Wako, Osaka, Japan) and stored the purified total RNA at −80 °C. Total RNA (500 ng) was reverse transcribed at 42 °C for 30 min. Amplification with specific primers for *MDR-1* and *β-actin* was performed in an iCycler (Bio-Rad) for 30 cycles of 45 s at 94 °C for denaturing, 45 s at 55 °C for annealing, and 90 s at 72 °C for extension. The primer sequences were as follows: human *β-actin* forward, 5′-TGAA-CCCCAAGGCCAACCGC-3′, reverse, 5′-TTGTGCTGGGT-GCCAGGGCA-3′; human *MDR-1* forward, 5′-CCCATCATT-GCAATAGCAGG-3′, reverse, 5′-GTTCAAACTTCTGCTC-CTGA-3′. Amplified products were electrophoresed with Marker 4 (Nippon Gene, Tokyo) on 3% agarose gels.

Dexamethasone Accumulation—[^{14}C]1-Butanol (Toho Biochemical, Tokyo; 1.61 mCi/mmol) was diluted with unlabeled butanol (Sigma) at a concentration of 0.5 MBq/ml. [^{3}H]Dexamethasone (PerkinElmer Life Sciences; 40.0 Ci/mmol) was dissolved in dimethyl sulfoxide (Me_2SO; Nacalai tesque, Tokyo) and then diluted with phosphate-buffered saline (final concentration of Me_2SO = 0.1%). CD4+ T cells, which were incubated with or without 50 μg/ml 6.9-kDa fragmented hyaluronan for 6 h at 37 °C, were suspended in phosphate-buffered saline with 7 mM dextrose for ATP supply, which is dispensable in this assay (35), at a density of 5×10^6 cells/ml. The CD4+ T cells were then incubated with 5.0×10^{-5} M [^{14}C]1-butanol and 3.0×10^{-8} M [^{3}H]dexamethasone for 20 min at 37 °C.

For competitive studies with cyclosporin A, CD4+ T cells were incubated with 100 ng/ml cyclosporin A (Novartis Pharmaceutical, Japan Co., Tokyo) for 15 min and then incubated with [^{14}C]1-butanol and [^{3}H]dexamethasone. Cyclosporin A was dissolved in Me_2SO before diluting with phosphate-buffered saline (final concentration of Me_2SO was 0.03%). After incubation with fragmented hyaluronan and cyclosporin A, 100 μg/ml aliquots were layered on 80 μl of the mixture of lauryl bromide and silicon oil (mixture ratio 2:1, Nacalai tesque, Tokyo) in an Eppendorf tube (Assist, Tokyo). After centrifugation at 10,000 × *g* for 2 min, the aliquots were rapidly frozen in liquid nitrogen, and the frozen tube was cut at the medium-mixture bound-arias, thereby obtaining the upper layer as the medium fraction and the lower layer as the cell fraction.

The obtained cell fractions were melted with soluene-350 and 10 ml of HIONIC-FLUOR (PerkinElmer) was added. The medium fractions were mixed with 10 ml of mixtures of toluene (Wako, Osaka, Japan), methanol (Wako), ethyleneglycol monoethyl ether (Nacalai tesque), and PERMAFLUOR (Packard; mixture ratio 200:50:50:12). The radioactivity of each fraction was counted with a scintillation counter. The cell to medium ratio (C/M ratio), which is an index of intracellular and extracellular dexamethasone concentration ratio, was computed using the following formula: C/M ratio = [(^{3}H in cell fraction/^{14}C in cell fraction)/(^{3}H in medium fraction/^{14}C in medium fraction)].

CD44 Blocking Analysis—CD4+ cells were incubated with 10 μg/ml Hermes-1 or rat IgG_{2a} as control mAb for 30 min at 37 °C prior to incubation with or without 50 μg/ml 6.9-kDa fragmented hyaluronan, the cells were used for the following experiments.

Western Blot Analysis—After incubation with or without 50 μg/ml 6.9-kDa fragmented hyaluronan, 2×10^6 of CD4+ T cells were homogenized in the presence of a hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). The cells were allowed to swell on ice for 15 min, after which 0.5% (v/v) Nonidet P-40 was added and vortexed. The homogenate was centrifuged at 20,000 × *g* to obtain pellet containing membrane. The pellet containing equal amounts of proteins prepared from 2×10^6 of CD4+ T cells were subjected to SDS-PAGE. The separated proteins were transferred onto the nitrocellulose membrane (Schleicher & Schuell). After blocking with TBST-milk (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% (v/v) Tween 20, and 5% nonfat dry milk), the membrane was incubated overnight with 10 μg/ml F4 at 4 °C. The membrane was then washed with TBST and incubated with horseradish peroxidase-conjugated anti-mouse mAb (1:1000 dilution in TBST) for 30 min at room temperature. After three washes in TBST, proteins were detected using a chemiluminescence reagent (Detection Reagent, Amersham Biosciences) following the procedure recommended by the manufacturer.

Statistical Analysis—Values are expressed as mean ± S.D. Student's *t* test was used to compare data between two groups. One-way analysis of variance and Bonferroni correction were used to compare data between three or more groups. $p < 0.05$ was considered statistically significant.

RESULTS

Fragmented Hyaluronan Up-regulates MDR-1 Gene Expression on PBMCs—We first assessed the effect of hyaluronan on P-glycoprotein expression in PBMCs. Expression of P-glycoprotein, as recognized by MRK16, was significantly induced by the addition of the 6.9-kDa fragmented hyaluronan during the observation periods (0–6 h), particularly at 6 h after incubation. In contrast, native hyaluronan (950 kDa) failed to induce P-glycoprotein expression on PBMCs (Fig. 1*A*). Therefore, we evaluated the expression of P-glycoprotein after a 6-h incubation with 6.9-kDa fragmented hyaluronan in the following studies. We observed that expression of P-glycoprotein was augmented in a dose-dependent manner up to 50 μg/ml 6.9-kDa fragmented hyaluronan (Fig. 1, *B* and *C*).

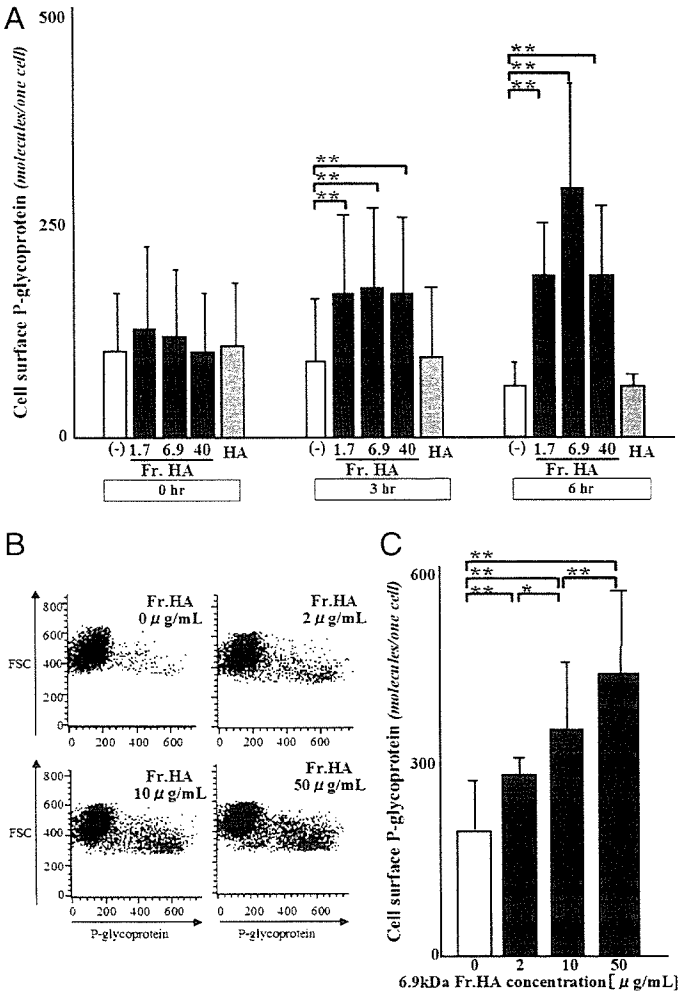

FIGURE 1. **Fragmented hyaluronan induces cell surface P-glycoprotein expression on PBMCs.** *A*, flow cytometric analysis showed P-glycoprotein expression on 1 × 10^6 of PBMCs after 0–6 h incubation with or without 10 μg/ml native (*HA*) or fragmented hyaluronan (*Fr. HA*). The molecular masses of fragmented hyaluronan were 1.7, 6.9, and 40 kDa. *B* and *C*, flow cytometric analysis showed P-glycoprotein expression on PBMCs after a 6-h incubation with different concentrations of 6.9-kDa fragmented hyaluronan. Each value represents the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Data represent mean ± S.D. of 10 independent experiments. Statistical analysis was performed using the paired *t* test. *, $p < 0.05$; **, $p < 0.01$.

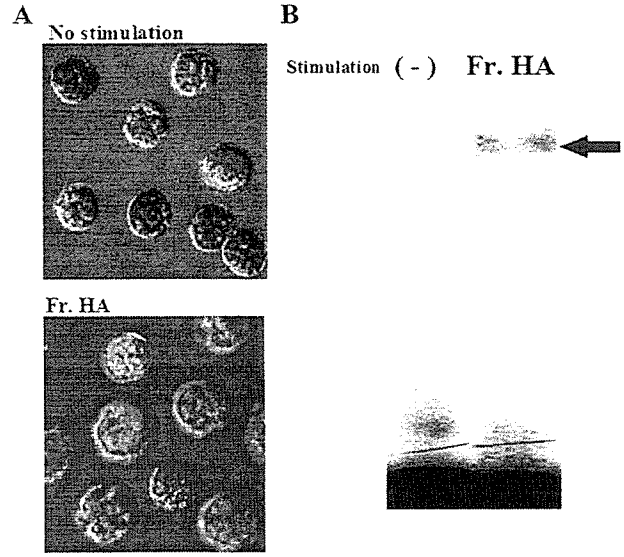

FIGURE 2. **Fragmented hyaluronan induces activation and nuclear translocation of YB-1 in PBMCs.** *A*, immunostaining and confocal microscopy analysis of YB-1 in 1 × 10^5 of PBMCs. YB-1 was expressed in the cytoplasm of all PBMCs without stimulation (*No stimulation*). In contrast, nuclear translocation of YB-1 was induced in 30% or more of PBMCs incubated with 50 μg/ml 6.9-kDa fragmented hyaluronan for 60 min at 37 °C (*Fr. HA*). Magnification, ×600. *B*, the YB-1 DNA binding activity was examined by electrophoretic mobility shift assay. Stimulation (60 min) with 50 μg/ml 6.9-kDa fragmented hyaluronan induced YB-1 DNA binding activity (*black arrow* indicates complex of YB-1/DNA).

Fragmented Hyaluronan Induces Nuclear Translocation of YB-1 in PBMCs—MDR-1 gene expression is initiated by activation and nuclear translocation of YB-1 in human lymphocytes (12). We examined the intracellular distribution of the transcriptional factor YB-1 in PBMCs by immunostaining using anti-YB-1 monoclonal antibody. Confocal microscopic analysis showed localization of YB-1 in the cytoplasm of PBMCs at basal conditions and its translocation into the nucleus within 60 min of incubation with 6.9-kDa fragmented hyaluronan (Fig. 2*A*). We confirmed the induction of the DNA binding activity of YB-1 by 6.9-kDa fragmented hyaluronan by electrophoretic mobility shift assay. Subsequently, we prepared nuclear extracts from PBMCs at basal conditions and after incubation with 6.9-kDa fragmented hyaluronan, and incubated them with a ^{32}P-labeled oligonucleotide containing YB-1 consensus binding sites. We observed dense bands in the mixture of oligonucleotides and nuclear extracts derived from PBMCs stimulated with 6.9-kDa fragmented hyaluronan (Fig. 2*B*). These results imply that incubation of PBMCs with 6.9-kDa fragmented hyaluronan resulted in a sequence of events including activation of cytoplasmic nuclear factor YB-1, its nuclear translocation, and finally the binding of activated YB-1 to the promoter region of the *MDR-1* gene. These results were consistent with those observed in the expression of P-glycoprotein in immunostaining.

YB-1 Antisense Inhibits P-glycoprotein Expression on Lymphocytes—To determine whether YB-1 is directly coupled with fragmented hyaluronan-induced *MDR-1* gene activation, we assessed the expression of P-glycoprotein on PBMCs transfected with *YB-1* antisense expression plasmid (PRC/CMV AS) or control vacant vector and compared the levels of P-glycoprotein expression on PBMCs incubated with or without 6.9-kDa fragmented hyaluronan. In comparison with the vector alone, introduction of *YB-1* antisense significantly reduced the expression of P-glycoprotein on PBMCs. Fragmented hyaluronan stimulation significantly increased the expression of P-glycoprotein on control cells, but such induction was abolished by transfection of YB-1 antisense (Fig. 3).

Fragmented Hyaluronan Up-regulation of P-glycoprotein Expression on CD4+ T Cells—To investigate the expression of P-glycoprotein on lymphocytes in more detail, we next performed two-color analysis using anti-CD4 and -CD8 antibodies and examined P-glycoprotein expression on each subset of lymphocytes. Flow cytometric analysis showed that P-glycoprotein expression was significantly augmented by 6.9-kDa fragmented hyaluronan on CD4+ but not CD8+ cells in PBMCs (Fig. 4*A*). Therefore, in further studies, we analyzed purified CD4+ T

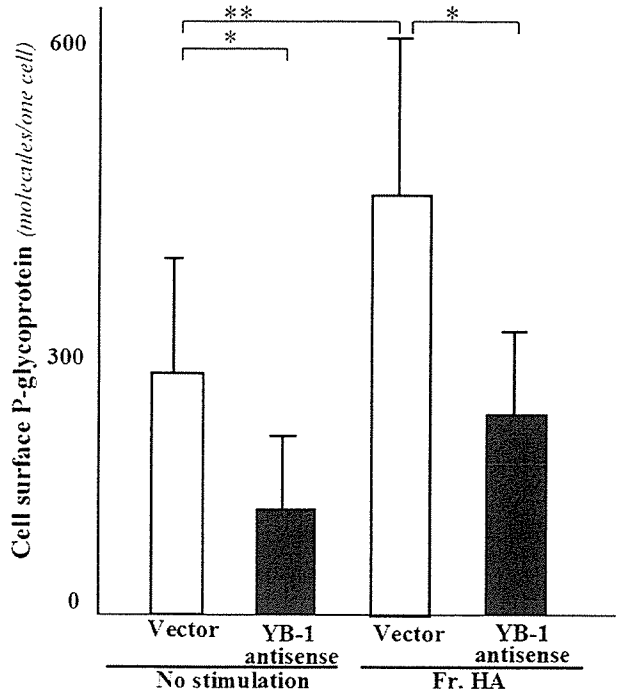

FIGURE 3. ***YB-1* antisense inhibits fragmented hyaluronan-induced P-glycoprotein expression on PBMCs.** Flow cytometric analysis showed P-glycoprotein expression on 2×10^6 of normal PBMCs that were transfected with *YB-1* antisense constructs (*closed bars*) or control vacant vector (*open bars*) and then incubated with or without 50 μg/ml 6.9-kDa fragmented hyaluronan (*Fr. HA*). Each value represents the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Data represent mean ± S.D. of five independent experiments. Statistical analysis was performed using the paired *t* test. *, $p < 0.05$; **, $p < 0.01$.

cells isolated from PBMCs. We first evaluated the expression of P-glycoprotein on isolated CD4+ T cells after a 6-h incubation with 6.9-kDa fragmented hyaluronan. Expression of P-glycoprotein on isolated CD4+ T cells was significantly induced by the addition of the fragmented hyaluronan (Fig. 4, *B–D*). To test whether P-glycoprotein expression was the result of *MDR-1* gene expression in response to fragmented hyaluronan, we examined the expression of *MDR-1* mRNA by reverse transcription-polymerase chain reaction. There was a substantial increase in *MDR-1* mRNA relative to *β-actin* mRNA in isolated CD4+ T cells incubated with 6.9-kDa fragmented hyaluronan, compared with CD4+ T cells at the basal condition (Fig. 4*E*).

Fragmented Hyaluronan Induces Simultaneous Activation of Resting CD4+ T Cells and Expression of P-glycoprotein—Recent studies from our laboratories demonstrated that soluble factors, which activate lymphocytes such as IL-2, induce YB-1 activation followed by P-glycoprotein expression in lymphocytes (13). In the present study, we evaluated whether up-regulation of P-glycoprotein expression by fragmented hyaluronan accorded with activation of lymphocytes. We used the cell surface maker CD69, a well defined early activation marker of T cells (36), to monitor CD4+ T cell activation. Flow cytometric analysis showed that CD69 expression on CD4+ T cells was significantly augmented by the 6.9-kDa fragmented hyaluronan (Fig. 5*A–D*). Furthermore, expression of P-glycoprotein was observed on CD69 high expressing CD4+ T cells, and the level of P-glycoprotein expression on CD69 high expressing CD4+ T cells was significantly augmented by the 6.9-kDa fragmented hyaluronan (Fig. 5, *E* and *F*). These results imply that expression of P-glycoprotein is closely associated with activation of CD4+ T cells and that up-regulation of P-glycoprotein by the 6.9-kDa fragmented hyaluronan occurs exclusively on CD69 high expressing CD4+ T cells.

Expression of P-glycoprotein Induces Excretion of Intracellular Dexamethasone in CD4+ T Cells—To investigate the association between expression of P-glycoprotein on CD4+ T cell and exclusion of drugs through P-glycoprotein, the intracellular and extracellular concentration of dexamethasone was determined. Stimulation with fragmented hyaluronan resulted in a significant decrease of intracellular dexamethasone of CD4+ T cells (Fig. 6). To confirm the functional involvement of P-glycoprotein in the decrease of intracellular dexamethasone, we added 100 ng/ml cyclosporin A, a competitive inhibitor of P-glycoprotein, to fragmented hyaluronan-stimulated CD4+ T cells. Excretion of dexamethasone in CD4+ T cells was completely inhibited by 100 ng/ml cyclosporin A (Fig. 6).

Anti-CD44 mAb Hermes-1 Inhibits Up-regulation of MDR-1 Gene Expression in CD4+ T Cells—One of the principal cellular receptors for hyaluronan is CD44 (19). Therefore, we used Hermes-1, a mAb blocking CD44, to investigate whether the CD44s epitope was essential for up-regulation of *MDR-1* gene expression in CD4+ T cells by fragmented hyaluronan. We found that fragmented hyaluronan markedly induced the nuclear translocation of YB-1 and the expression of cell surface P-glycoprotein by immune staining and Western blotting analysis. The inducibility was abolished by preincubation with Hermes-1, confirming the involvement of signal thorough CD44 molecules in fragmented hyaluronan-induced P-glycoprotein expression (Fig. 7).

DISCUSSION

In terms of interaction of hyaluronan with cell surface CD44, three functional categories of CD44 have been reported: non-binding CD44, non-binding CD44 unless activated, and constitutively active CD44. Most cells of connective tissue, as well as some proliferative epithelial cells and tumor cells appear to express constitutively active CD44. In contrast, CD44 is functionally inactive on resting T cells, and is not able to bind hyaluronan without external stimuli such as TCR stimulation (37). Fragmented hyaluronan but not native hyaluronan has been shown to stimulate the expression of cytokines, proinflammatory chemokines, and cell adhesion molecules possibly through a mechanism involving CD44 in endothelial cells, various malignant cells, and macrophages (24–26).

In this report, we demonstrate a novel physiological function of fragmented hyaluronan on CD4+ T cells. The main findings of the present study were as follows. 1) Fragmented hyaluronan, but not the native high molecular weight hyaluronan, was able to induce transcriptional regulation of the *MDR-1* gene, YB-1 activation followed by P-glycoprotein expression, in CD4+ T cells. 2) Expression of P-glycoprotein on CD4+ T cells induced by fragmented hyaluronan was observed preferentially on activated CD4+ T cells. 3) Overexpression of P-glycoprotein resulted in dexamethasone excretion from CD4+ T cells, which was inhibited by the competitive inhibitor cyclosporin A. 4) Transcriptional induction of *MDR-1* gene by fragmented

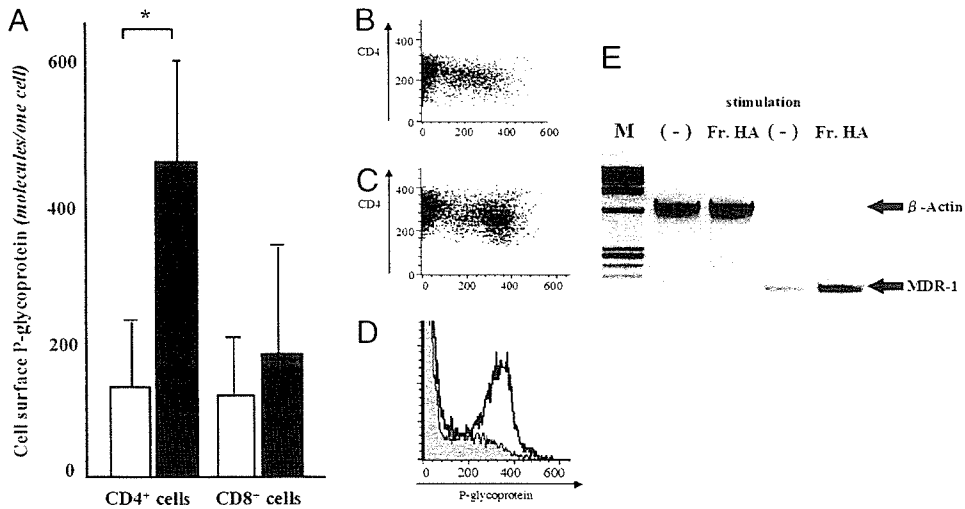

FIGURE 4. **Fragmented hyaluronan induces expression of P-glycoprotein on CD4+ T cells.** *A*, flow cytometric analysis showed P-glycoprotein expression on peripheral CD4+ and CD8+ T cells in 1×10^6 of PBMCs in six independent donors incubated with (*solid bars*) or without (*open bars*) 50 μg/ml 6.9-kDa fragmented hyaluronan for 6 h. Each value represents the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Data represent mean ± S.D. of six independent experiments. Statistical analysis was performed using the paired *t* test. *, $p < 0.05$. *B–D*, flow cytometric analysis showed P-glycoprotein expression on 5×10^5 of CD4+ T cells isolated from PBMCs (*B* and *gray area* in *D*) increased after 6 h stimulation with 50 μg/ml 6.9-kDa fragmented hyaluronan (*C* and *solid black line* in *D*). *E*, *MDR-1* mRNA expression was examined by reverse transcriptase-PCR using total RNA extracted from 1×10^6 of CD4+ T cells isolated from PBMCs incubated with or without 50 μg/ml 6.9-kDa fragmented hyaluronan (*Fr. HA*) for 6 h. β-Actin (β-actin) transcript was used as an internal standard. *M*, molecular size marker φX 174 HaeIII digestion.

hyaluronan was mediated through CD44 molecules on CD4+ T cells.

Other investigators have reported that fragmented hyaluronan binding to cell surface CD44 progressively increased in avidity with increasing oligomer size from 20-mers to 38-mers, and that fragmented hyaluronan (38), especially the 6.9-kDa fragmented hyaluronan (36-mers), enhanced CD44 cleavage as well as promoted tumor cell mobility without certain stimuli (*e.g.* phorbol ester and anti-CD44 stimulating antibodies *in vitro*), whereas a large polymer hyaluronan failed to induce them (39). This evidence was consistent with our results showing that 6.9-kDa fragmented hyaluronan induced expression of P-glycoprotein on lymphocytes most strongly.

In contrast to our results, Misra *et al.* (40) reported that fragmented hyaluronan reduces *MDR-1* expression in human breast carcinoma cells. The reasons for the conflicting result on the function of fragmented hyaluronan are due, at least, to the difference of oligomer size of fragmented hyaluronan. They used oligomers of 3–10-mers that could do only monovalent binding, whereas we used oligomers of 36-mers, which allow at least divalent binding (38). The oligomers of 3–10-mers antagonize constitutive interaction between hyaluronan polymer and CD44 by competitively replacing, because multivalent interaction can transduce signals although a monovalent interaction does not (40). On the other hand, we showed that the oligomers of 36-mers induce signal transduction after interaction with CD44 molecules on the resting CD4+ T cells and that the interaction induces activation of CD4+ T cells and expression of MDR1 mRNA. Others reported that 4–6-mers induced maturation of dendritic cells without CD44 (41). Therefore, different sizes of hyaluronan oligomers might induce the different effects on CD44-hyaluronan interaction.

In addition to hyaluronan oligomers, different sizes of hyaluronan polymers might have different biological activities. Breast cancer cells express both hyaluronan synthase and hyaluronidase, so they rapidly synthesize high molecular hyaluronan while simultaneously degrading it into small and intermediate sized polymers (20–500 kDa) (42). These small or intermediate sized polymers could induce *MDR-1* expression in human breast carcinoma cells (40). We also demonstrated that small sized polymers (40 kDa) could induce expression of P-glycoprotein on lymphocytes. However, the large sized polymers (probably ~600 kDa), which include our native hyaluronan (950 kDa), could not induce *MDR-1* expression. The large sized polymers also have anti-angiogenic (43), anti-inflammatory, and immunosuppressive effects (44, 45), and they do not induce CD44 cleavage (39).

Therefore, hyaluronan changes activities of *MDR-1* induction, as well as other biological activities, depending on its molecular weight. The oligomers of 3–10-mers (probably 4–6-mers, 0.8–1.2 kDa) inhibit *MDR-1* expression competitively by monovalent interaction with CD44. The larger oligomers (~1.7 kDa), small and intermediate sized polymers (~500 kDa) could induce *MDR-1* expression by more than the divalent interaction with CD44, and the oligomers of 36-mers (6.9 kDa) might be the most potent activators of *MDR-1* expression. The large sized polymers (probably ~600 kDa) could not induce *MDR-1* expression.

Furthermore, the manner of hyaluronan production is different between resting CD4+ T cells and human breast carcinoma cells. Although CD4+ T cells constitutively express mRNA for hyaluronan synthases, their surface hyaluronan expression becomes detectable only after cell activation. In contrast, human breast carcinoma cells spontaneously produce hyaluronan without any stimulation, express constitutively active CD44, but CD44 on resting T cells is functionally inactive and cannot bind hyaluronan without external stimuli such as stimulation (37). Thus, unless CD4+ T cells are stimulated, they express neither *MDR-1*, the active form of CD44, nor cell surface hyaluronan, whereas on human breast carcinoma cells *MDR-1* could be constitutively expressed through endogenous small and intermediate sized hyaluronan polymers by CD44-mediated self-activation. We, therefore, document that 6.9-kDa fragmented hyaluronan (36-mers) interacts with CD44 on the resting CD4+ T cells, and that the interaction induces activation of CD4+ T cells and induction of *MDR-1* expression.

Fragmented hyaluronan can be generated *in vivo* by various mechanisms, such as enzymatic digestion by hyaluronidases and acid hydrolases, degradation by oxygen-derived free radi-

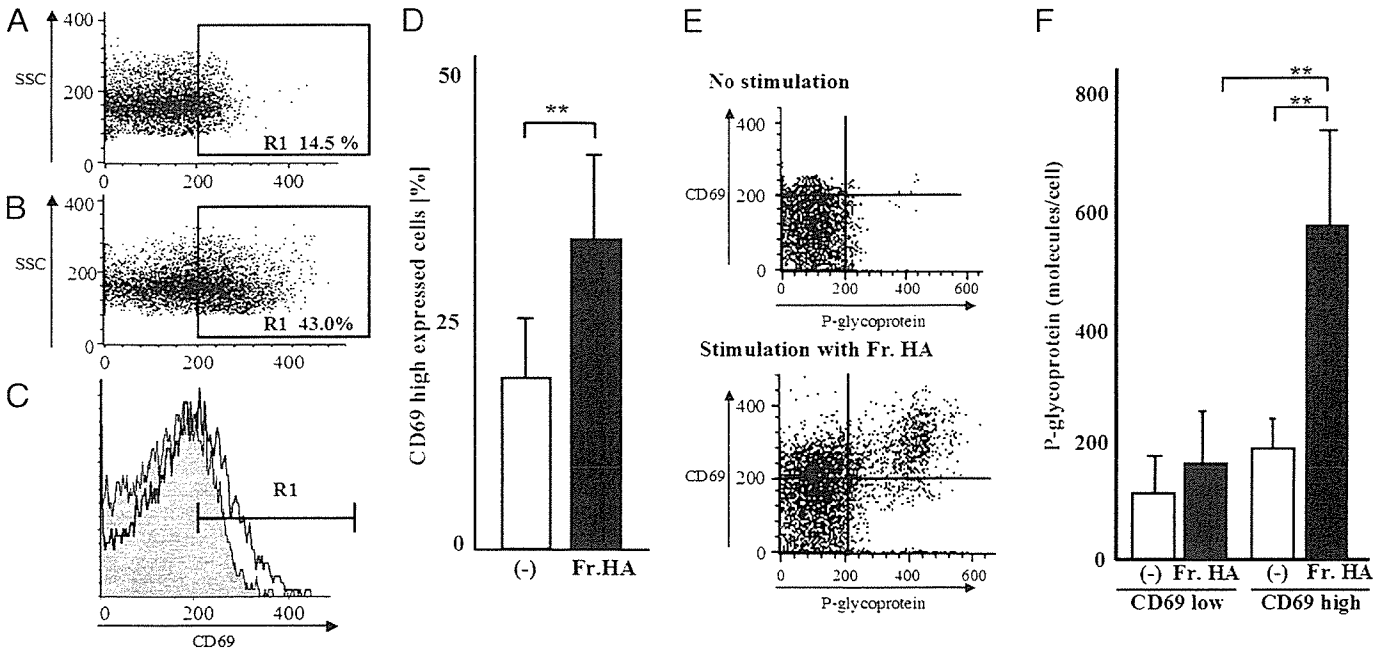

FIGURE 5. **Fragmented hyaluronan induces activation of CD4+ T cells, which results in the expression of P-glycoprotein.** *A–C*, flow cytometric analysis showed increased expression of CD69 on 5×10^5 of CD4+ T cells isolated from PBMCs (*A* and *gray area* in *C*) at 3 h after stimulation with 50 μg/ml 6.9-kDa fragmented hyaluronan (*B* and *solid black line* in *C*). The region R1 indicates CD69 high expressing cells. *D*, data represent the percentage of CD69 high expressing cells among 5×10^5 of CD4+ T cells isolated from PBMCs from 10 independent donors incubated with (*solid bars*) or without (*open bars*) 50 μg/ml 6.9-kDa fragmented hyaluronan for 3 h. Data represent mean ± S.D. of 10 independent experiments. Statistical analysis was performed using the paired *t* test. *, $p < 0.05$. *E*, flow cytometric analysis showed P-glycoprotein expression on CD69 high or low expressing CD4+ T cells after a 6-h incubation with or without 50 μg/ml 6.9-kDa fragmented hyaluronan (*Fr. HA*). *F*, P-glycoprotein expression on CD69 high or low expressing CD4+ T cells after a 3-h incubation with (*closed bars*) or without (*open bars*) 50 μg/ml 6.9-kDa fragmented hyaluronan (*Fr. HA*). Each value represents the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Data represent mean ± S.D. of 10 independent experiments. Statistical analysis was performed using the paired *t* test. *, $p < 0.05$.

cals, or *de novo* synthesis of low molecular mass hyaluronan (46, 47). Fragmented hyaluronan exists in the place of tumor invasion or inflammation, and closely relates to their pathogenesis (21–24).

In rheumatoid arthritis (RA), which is characterized by progressive polyarthritis with occasional extraarticular involvement, fragmented hyaluronan is increased in the RA synovium and synovial fluid (21, 48). Although the production of native hyaluronan by RA synovial cells is promoted according to the aggravation of inflammation by inflammatory cytokines including IL-1β and tumor necrosis factor-α (49), the activity of hyaluronidase produced by CD14+ monocytes is so high that native hyaluronan is digested to fragmented hyaluronan immediately at the loci of inflammation (50). Furthermore, the production of fragmented hyaluronan is accelerated due to oxygen-derived free radicals in the inflammation locus like synovitis (21, 48). Our previous studies showed that fragmented hyaluronan, which is increased by inflammation in proportion to RA disease activity, induced the expression of vascular cell adhesion molecule-1 on synovial cells and is involved in the pathogenesis of RA (51, 52).

Recently, we found that IL-2, a potent stimulus of lymphocytes (53, 54), up-regulated P-glycoprotein expression on lymphocytes via activation of transcriptional factor YB-1 and that such up-regulation markedly reduced intracellular corticosteroid concentration *in vitro* (13). In this study, we found that 6.9-kDa fragmented hyaluronan was also a potent stimulus of CD4+ T cells as well as IL-2. We demonstrated that 6.9-kDa fragmented hyaluronan could activate resting CD4+ T cells and induce P-glycoprotein expression simultaneously without any other co-stimuli.

We propose that the expression of P-glycoprotein in accordance with activation of CD4+ T cells is a serious problem in autoimmune diseases including RA and systemic lupus erythematosus. Autoimmune diseases are characterized by inflammation induced by activation of autoreactive T cells and production of autoantibodies from activated B cells, which is enhanced by intercellular adhesion, adhesion to extracellular matrix, and cytokines such as IL-2 (51–55). The main strategy for treatment is to control such autoreactive lymphocytes with corticosteroids, disease-modifying antirheumatic drugs, and other immunosuppressants (56). Corticosteroids, certain immunosuppressive agents, and disease-modifying antirheumatic drugs, including chloroquine and sulfasalazine, are extruded from lymphocytes by P-glycoprotein (3, 13, 57). We note that P-glycoprotein acts as a "hydrophobic vacuum cleaner," *i.e.* P-glycoprotein catches drugs like a vacuum cleaner when they pass through the cell membrane, and then releases them outside the cell. Substrates of P-glycoprotein, including corticosteroids and some disease-modifying antirheumatic drugs, cannot reach the cytoplasm by increasing the number of P-glycoproteins on lymphocytes, thus resulting in treatment failure. Indeed, our group and others have reported that low cytoplasmic corticosteroid concentrations, caused by increased P-glycoprotein-mediated efflux of corticosteroids from lymphocytes, is one of the mechanisms of corticosteroid resistance in inflammatory bowel disease, asthma, and systemic lupus erythematosus (58–60). Furthermore, the expression of P-glyco-

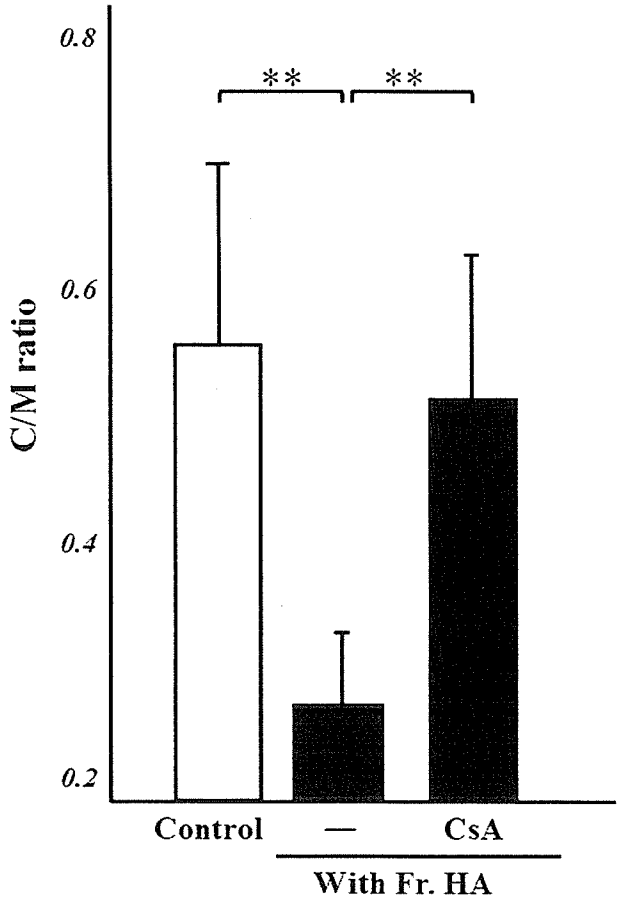

FIGURE 6. **Excretion of intracellular dexamethasone through P-glycoprotein induced by fragmented hyaluronan.** CD4+ T cells were preincubated with (*solid bars*) or without (*open bar*) 50 μg/ml 6.9-kDa fragmented hyaluronan (*Fr. HA*) for 6 h. Then 20 min after the addition of [6,7-^{3}H]dexamethasone, the C/M ratio was evaluated in the presence or absence of 100 ng/ml cyclosporin A. Data represent mean ± S.D. of 10 independent experiments. Statistical analysis was performed using the paired *t* test. **, $p < 0.01$.

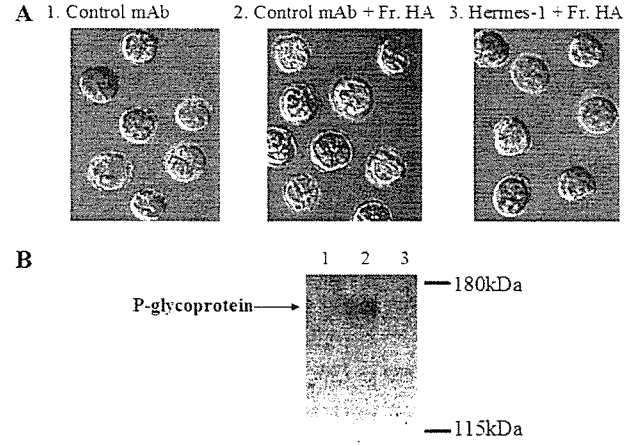

FIGURE 7. **Anti-CD44 antibody inhibits fragmented hyaluronan-induced *MDR-1* gene expression in CD4+ T cells.** *A*, immunostaining and confocal microscopy analysis of YB-1 in 1 × 10^5 of CD4+ T cells. CD4+ T cells were pretreated with anti-CD44 mAb Hermes-1 or with isotype control mAb at a concentration of 10 μg/ml. CD4+ T cells were then incubated with 50 μg/ml 6.9-kDa fragmented hyaluronan for 60 min at 37 °C (*Fr. HA*). Magnification, ×600. *B*, immunodetection of P-glycoprotein on Western blots of prepared CD4+ T cells. CD4+ T cells were pretreated with anti-CD44 mAb Hermes-1 or with isotype control mAb at a concentration of 10 μg/ml. CD4+ T cells were then incubated with 50 μg/ml 6.9-kDa fragmented hyaluronan (*Fr. HA*) for 6 h at 37 °C. Samples containing equal amounts of proteins prepared from 2 × 10^6 of CD4+ T cells were subjected to SDS-PAGE. Separated proteins were transferred onto nitrocellulose membranes and probed with anti-P-glycoprotein Ab (F4). *Lane 1*, control mAb only; *lane 2*, control mAb + Fr. HA; *lane 3*, Hermes-1 + Fr. HA.

protein on lymphocytes and the efficacy of cyclosporin A, a competitive inhibitor of P-glycoprotein, have been reported in refractory RA patients (61–63).

As another pathological relevance of hyaluronan, several studies have shown that fragmented hyaluronan strongly stimulates tumor invasion, growth, and P-glycoprotein-mediated multidrug resistance (22, 25, 40). Otherwise, our results suggest that the fragmented hyaluronan around tumors activates not only tumors but also CD4+ T cells of the host. Expression of P-glycoprotein on CD4+ T cells is induced in accordance with activating CD4+ T cells by fragmented hyaluronan around tumors, IL-2 and interferon-γ might be extruded through P-glycoprotein on CD4+ T cells (64), and then these cytokines might activate effector cells including LAK and NK resulting in enhancement of the anti-tumor effect (65). Furthermore, CD4+ T cells could survive by acquiring P-glycoprotein-mediated chemotherapy resistance. Thus, it will be advantageous for the host that expression of P-glycoprotein on CD4+ T cells is increased in accordance with activation of CD4+ T cells induced by fragmented hyaluronan around tumors.

In this study, we propose that the sequential events in fragmented hyaluronan-stimulated CD4+ T cells, consist of CD44 activation, *MDR-1* transcription, P-glycoprotein expression, and production of P-glycoprotein substrates that can be inhibited by P-glycoprotein competitors. Our findings suggest that a characteristic change of extracellular matrix in a specific environment could directly modulate the immune system. Our findings also expand our understanding of the interaction between tumors, chronic inflammation, and the immune system.

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REFERENCES

1. Linn, S. C., Honkoop, A. H., Hoekman, K., van der Valk, P., Pinedo [illegible] and Giaccone, G. (1996) *Br. J. Cancer* **74,** 63–68
2. Tsuruo, T. (1983) *Cancer Treat. Rep.* **67,** 889–894
3. Ueda, K., Cardaralli, C., Gottesman, M. M., and Pastan, I. [illegible] *Natl. Acad. Sci. U. S. A.* **84,** 3004–3008
4. Beck, W. T., Grogan, T. M., Willman, C. L., Cordon-Cardo, C., Parham, D. M., Kuttesch, J. F., Andreeff, M., Bates, S. E., Berard, C. W., Boyett, J. M., Brophy, N. A., Broxterman, H. J., Chan, H. S., Dalton, W. S., Dietel, M., Fojo, A. T., Gascoyne, R. D., Head, D., Houghton, P. J., Srivastava, D. K., Lehnert, M., Leith, C. P., Paietta, E., Pavelic, Z. P., and Weinstein, R. (1996) *Cancer Res.* **56,** 3010–3020
5. Advani, R., Visani, G., Milligan, D., Saba, H., Tallman, M., Rowe, J. M., Wiernik, P. H., Ramek, J., Dugan, K., Lum, B., Villena, J., Davis, E., Paietta, E., Litchman, M., Covelli, A., Sikic, B., and Greenberg, P. (1999) *Adv. Exp. Med. Biol.* **457,** 47–56
6. List, A. F., Kopecky, K. J., Willman, C. L., Head, D. R., Slovak, M. L., Douer, D., Dakhil, S. R., and Appelbaum, F. R. (2002) *Blood* **100,** 1910–1912
7. Chen, C. J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1986) *Cell* **47,** 381–389
8. Bourgeois, S., Gruol, D. J., Newby, R. F., and Rajah, F. M. (1993) *Mol. Endocrinol.* **7,** 840–851

9. Fojo, A. T., Ueda, K., Slamon, D. J., Poplack, D. G., Gottesman, M. M., and Pastan, I. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 265–269
10. Sugawara, I., Kataoka, I., Morishita, Y., Hamada, H., Tsuruo, T., Itoyama, S., and Mori, S. (1988) *Cancer Res.* **48,** 1926–1929
11. Cordon-Cardo, C., O'Brien, J. P., Casals, D., Rittman-Grauer, L., Biedler, J. L., Melamed, M. R., and Bertino, J. R. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 695–698
12. Chaudhary, P. M., and Roninson, I. B. (1991) *Cell* **66,** 85–94
13. Tsujimura, S., Saito, K., Nakayamada, S., Nakano, K., Tsukada, J., Kohno, K., and Tanaka, Y. (2004) *Genes Cells* **9,** 1265–1273
14. Ohga, T., Uchiumi, T., Makino, Y., Koike, K., Wada, M., Kuwano, M., and Kohno K. (1998) *J. Biol. Chem.* **273,** 5997–6000
15. Kohno, K., Sato, S., Takano, H., Matsuo, K., and Kuwano, M. (1989) *Biochem. Biophys. Res. Commun.* **165,** 1415–1421
16. Sturm, A., Krivacic, K. A., Fiocchi, C., and Levine, A. D. (2004) *J. Immunol.* **173,** 3889–3900
17. Shimizu, Y., and Shaw, S. (1991) *FASEB J.* **5,** 2292–2299
18. Sugahara, K., Schwartz, N. B., and Dorfman, A. (1979) *J. Biol. Chem.* **254,** 6252–6261
19. Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B., and Seed, B. (1990) *Cell* **61,** 1303–1313
20. Underhill, C. (1992) *J. Cell Sci.* **103,** 293–298
21. Balazs, E. A., Watson, D., Duff, I. F., and Roseman, S. (1967) *Arthritis Rheum.* **10,** 357–376
22. Liu, D., Pearlman, E., Diaconu, E., Guo, K., Mori, H., Haqqi, T., Markowitz, S., Willson, J., and Sy, M. S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7832–7837
23. Lokeshwar, V. B., Rubinowicz, D., Schroeder, G. L., Forgacs, E., Minna, J. D., Block, N. L., Nadji, M., and Lokeshwar, B. L. (2001) *J. Biol. Chem.* **276,** 11922–11932
24. Deed, R., Rooney, P., Kumar, P., Norton, J. D., Smith, J., Freemont, A. J., and Kumar, S. (1997) *Int. J. Cancer* **71,** 251–256
25. Rooney, P., Kumar, S., Ponting, J., and Wang, M. (1995) *Int. J. Cancer* **60,** 632–636
26. McKee, C. M., Penno, M. B., Cowman, M., Burdick, M. D., Strieter, R. M., Bao, C., and Noble, P. W. (1996) *J. Clin. Investig.* **98,** 2403–2413
27. Tanaka, Y., Minami, Y., Mine, S., Hirano, H., Hu, C. D., Fujimoto, H., Fujii, K., Saito, K., Tsukada, J., van Kooyk, Y., Figdor, C. G., Kataoka, T., and Eto, S. (1999) *J. Immunol.* **163,** 6209–6216
28. Tanaka, Y., Wake, A., Horgan, K. J., Murakami, S., Aso, M., Saito, K., Oda, S., Morimoto, I., Uno, H., Kikuchi, H., Izumi, Y., and Eto, S. (1997) *J. Immunol.* **158,** 3822–3829
29. Tanaka, Y., Kimata, K., Wake, A., Mine, S., Morimoto, I., Yamakawa, N., Habuchi, H., Ashikari, S., Yamamoto, H., Sakurai, K., Yoshida, K., Suzuki, S., and Eto, S. (1996) *J. Exp. Med.* **184,** 1987–1997
30. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489
31. Ohga, T., Koike, K., Ono, M., Makino, Y., Itagaki, Y., Tanimoto, M., Kuwano, M., and Kohno, K. (1996) *Cancer Res.* **56,** 4224–4228
32. Itoh, H., Mukoyama, M., Pratt, R. E., Gibbons, G. H., and Dzau, V. J. (1993) *J. Clin. Investig.* **91,** 2268–2274
33. Rodriguez-Viciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997) *Cell* **89,** 457–467
34. Tamada, M., Hu, C. D., Kariya, K., Okada, T., and Kataoka, T. (1997) *Oncogene* **15,** 2959–2964
35. Richard, C., and John, R. R. (1993) *J. Biol. Chem.* **268,** 16059–16064
36. Ziegler, S. F., Ramsdell, F., and Alderson, M. R. (1994) *Stem Cells (Dayton)* **12,** 456–465
37. DeGrendele, H. C., Kosfiszer, M., Estess, P., and Siegelman, M. H. (1997) *J. Immunol.* **159,** 2549–2553
38. Lesley, J., Hascall, V. C., Tammi, M., and Hyman, R. (2000) *J. Biol. Chem.* **275,** 26967–26975
39. Sugahara, K. N., Murai, T., Nishinakamura, H., Kawashima, H., Saya, H., and Miyasaka, M. (2003) *J. Biol. Chem.* **278,** 32259–32265
40. Misra, S., Ghatak, S., and Toole, B. P. (2005) *J. Biol. Chem.* **280,** 20310–20315
41. Termeer, C. C., Hennies, J., Voith, U., Ahrens, T., Weiss, J. M., Prehm, P., and Simon, J. C. (2000) *J. Immunol.* **165,** 1863–1870
42. Udabage, L., Brownlee, G. R., Nilsson, S. K., and Brown, T. J. (2005) *Exp. Cell Res.* **310,** 205–217
43. Feinberg, R. N., and Beebe, D. C. (1983) *Science* **220,** 1177–1179
44. Delmage, J. M., Powars, D. R., Jaynes, P. K., and Allerton, S. E. (1986) *Ann. Clin. Lab. Sci.* **16,** 303–310
45. McBride, W. H., and Bard, J. B. (1979) *J. Exp. Med.* **149,** 507–515
46. Noble, P. W. (2002) *Matrix Biol.* **21,** 25–29
47. Itano, N., Sawai, T., Yoshida, M., Lenas, P., Yamada, Y., Imagawa, M., Shinomura, T., Hamaguchi, M., Yoshida, Y., Ohnuki, Y., Miyauchi, S., Spicer, A. P., McDonald, J. A., and Kimata, K. (1999) *J. Biol. Chem.* **274,** 25085–25092
48. Dahl, L. B., Dahl, I. M., Engstrom-Laurent, A., and Granath, K. (1985) *Ann. Rheum. Dis.* **44,** 817–822
49. Wells, A. F., Klareskog, L., Lindblad, S., and Laurent, T. C. (1992) *Arthritis. Rheum.* **35,** 391–396
50. Nagaya, H., Ymagata, T., Ymagata, S., Iyoda, K., Ito, H., Hasegawa, Y., and Iwata, H. (1999) *Ann. Rheum. Dis.* **58,** 186–188
51. Fujii, K., Fujii, Y., Hubscher, S., and Tanaka, Y. (2001) *J. Immunol.* **167,** 1198–1203
52. Fujii, K., Tanaka, Y., Hubscher, S., Saito, K., Ota, T., and Eto, S. (1996) *J. Immunol.* **162,** 2391–2398
53. Kelly, E., Won, A., Refaeli, Y., and Van Parijs, L. (2002) *J. Immunol.* **168,** 597–603
54. Luxembourg, A. T., and Cooper, N. R. (1994) *J. Immunol.* **153,** 604–614
55. Choy, E. H., and Panayi, G. S. (2001) *N. Engl. J. Med.* **344,** 907–916
56. Hahn, B. H. (2001) *Harrison's Principles of Internal Medicine*, 15th Ed., McGraw-Hill, Columbus, OH
57. Salmon, S. E., and Dalton, W. S. (1996) *J. Rheumatol. Suppl.* **44,** 97–101
58. Tsujimura, S., Saito, K., Nakayamada, S., Nakano, K., and Tanaka, Y. (2005) *Arthritis Rheum.* **52,** 1676–1683
59. Farrell, R. J., and Kelleher, D. (2003) *J. Endocrinol.* **178,** 339–346
60. Montano, E., Schmitz, M., Blaser, K., and Simon, H. U. (1996) *J. Investig. Allerg. Clin. Immunol.* **6,** 14–21
61. Yudoh, K., Matsuno, H., Nakazawa, F., Yonezawa, T., and Kimura, T. (1999) *Arthritis Rheum.* **42,** 2014–2015
62. Proudman, S. M., Conaghan, P. G., Richardson, C., Griffiths, B., Green, M. J., McGonagle, D., Wakefield, R. J., Reece, R. J., Miles, S., Adebajo, A., Gough, A., Helliwell, P., Martin, M., Huston, G., Pease, C., Veale, D. J., Isaacs, J., van der Heijde, D. M., and Emery, P. (2000) *Arthritis Rheum.* **43,** 1809–1819
63. Gerards, A. H., Landewe, R. B., Prins, A. P., Bruyn, G. A., Goei The, H. S., Laan, R. F., and Dijkmans, B. A. (2003) *Ann. Rheum. Dis.* **62,** 291–296
64. Drach, J., Gsur, A., Hamilton, G., Zhao, S., Angerler, J., Fiegl, M., Zojer, N., Raderer, M., Haberl, I., Andreeff, M., and Huber, H. (1996) *Blood* **88,** 1747–1754
65. Frederick, M., Grimm, E., Krohn, E., Smid, C., and Yu, T. K. (1997) *J. Interferon Cytokine Res.* **17,** 435–437

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SPECIAL ARTICLE

International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS)

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Summary. New clinical, laboratory and experimental insights, since the 1999 publication of the Sapporo preliminary classification criteria for antiphospholipid syndrome (APS), had been addressed at a workshop in Sydney, Australia, before the Eleventh International Congress on antiphospholipid antibodies. In this document, we appraise the existing evidence on clinical and laboratory features of APS addressed during the forum. Based on this, we propose amendments to the Sapporo criteria. We also provide definitions on features of APS that were not included in the updated criteria.

Keywords: anticardiolipin, antiphospolipid syndrome, β_2 glycoprotein-I, classification criteria, lupus anticoagulant, thrombosis.

Introduction

Since the formulation of the international preliminary classification (Sapporo) criteria for antiphospholipid syndrome (APS) [1], a significant body of work in basic research and studies on laboratory and clinical manifestations of APS has appeared. A preconference workshop, preceding the Eleventh International Congress on antiphospholipid antibodies (aPL), considered revisions to the international classification criteria for APS. Members of the workshop panel included all of the authors and the individuals listed in the Appendix.

Some of the authors presented the current evidence in their area of expertise (see Addendum) providing relevant literature on predictors of outcome, risk factors, associations between clinical and laboratory features and accuracy of tests. The evidence was also reviewed and graded (according to criteria listed in Table 1) by three members of the committee (SM, MDL, SAK) not involved in the presentation of specific topics. An open discussion followed, to reach consensus. Where data were limited or incongruent, expert opinion supplements the recommendations, as indicated.

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Update of the classification criteria

Table 2 contains the revised classification criteria for APS. The Sapporo classification divided the APS criteria into clinical and laboratory; this categorization was maintained in the current revision.

Studies validating the Sapporo criteria [2,3] are few. Tested against patients with systemic lupus erythematosus (SLE) and lupus-like disease (LLD), the criteria have high sensitivity and specificity [2], but the high frequency of aPL in older populations and of thromboembolic disease in hospitalized patients suggests that the Sapporo criteria would perform poorly in these populations. The association of aging and of common risk factors for cardiovascular disease with thrombosis may cause classification bias (Evidence Level I) [4]. No published data provides a valid estimation of an age boundary for diagnosing APS. Standard definitions of premature cardiovascular disease [5] and conditions conferring risk for thrombosis (listed in Table 2) [6,7] should be taken into account (Evidence Level I). Thrombosis may be more frequent when multiple risk factors coexist. Strict exclusion criteria

Table 1 Classification of evidence used in this article for evaluating studies regarding the association of risk factors with clinical conditions and/or disease outcome*

Evidence Level	Description
Class I	Prospective study in a broad spectrum of the representative population or Meta-analysis of randomized-controlled trials
Class II	Prospective study in a narrow spectrum of the representative population or Well-designed cohort or case–control analytic study or Retrospective study in a broad spectrum of the representative population
Class III	Retrospective study in a narrow spectrum of the representative population
Class IV	Study design where predictor is not applied in a blinded fashion or Descriptive case series or Expert opinion

*Throughout this article wherever studies of different Evidence Levels are quoted for the same issue, only the higher Evidence Level is provided.

therefore seem impractical. The committee concurs that additional factors contributing to thrombosis should be assessed and that APS patients should be stratified according to: (a) the presence or (b) absence of other – inherited or acquired – contributing causes of thrombosis (Table 2).

Evidence arising from clinical experience and the few relevant publications suggests that the 'fetal death' (Type 2a) Sapporo pregnancy morbidity criterion is the most specific, while the 'recurrent early abortion' (Type 2c) criterion may be the most sensitive (Level of evidence IV). The specificity of recurrent early abortion is uncertain because of the difficulty in excluding other known or suspected causes. The pre-eclampsia/placental insufficiency (Type 2b) Sapporo criterion may be relatively insensitive or non-specific. To enhance specificity, this criterion included only cases requiring delivery before 34 weeks' gestation. It appears that some investigators have incorrectly interpreted this criterion to include any preterm birth because of pre-eclampsia or placental insufficiency. In (small) populations unselected [8] or at risk for recurrent pre-eclampsia [9], aPL are not associated with pre-eclampsia or placental insufficiency (Evidence Level II). We recognize that there is no widely accepted definition for placental insufficiency, that timing of delivery is subject to physician judgment, and that there are no specific histopathologic placental abnormalities characteristic of either APS or 'severe' placental insufficiency (Evidence Level III) [10]. Well-designed, prospective studies to determine the contribution of APS to the overall problem of preterm birth from severe pre-eclampsia or placental insufficiency are not available. The committee finds no advantage to removing the pre-eclampsia/placental insufficiency criterion; there is a need for optimizing its performance instead. We recommend adherence to strict definitions of eclampsia and severe pre-eclampsia [11,12], we provide the commonly used clinical definitions for placental insufficiency (Table 2), and we suggest that the criterion for APS classification be any of these conditions associated with the decision of a qualified clinician to deliver a morphologically normal fetus prior to 34 weeks' gestation.

Both lupus anticoagulant (LA) and anticardiolipin (aCL) immunoglobulin isotype G (IgG) and M (IgM) are maintained as laboratory APS criteria, and IgG and IgM anti-β_2 glycoprotein-I (anti-β_2GPI) assays are added in the revised criteria (Table 2).

Medium and high titers of IgG and IgM aCL antibodies associate with clinical manifestations of APS, and were selected as criteria in Sapporo. However, the threshold used to distinguish moderate–high levels from low levels has no standard [13], and definition of the level that best corresponds to the risk of clinical manifestations is difficult [14]. Based on the best available evidence (Evidence Level II) [15–19], and until an international consensus is reached, the committee introduces a clear statement on threshold for positive: >40 GPL or MPL units, or >99th percentile (Table 2).

The revized criteria introduce a concept of subclassification of APS patients into four different categories of aPL assay positivity, specified in Table 2. Certain issues of specificity and predictive value of laboratory assays remain unresolved, whereas evidence suggests that multiple aPL positivity is associated with a more severe course of the disease, increasing significantly the rate of thrombosis (Evidence Level II) [20–23]. Investigators encouraged to subclassify patients with positive laboratory assays that fulfil the criteria for APS in clinical studies, according to the guidelines in Table 2.

Antiphospholipid syndrome requires the combination of at least one clinical and one laboratory criterion. A remote test avoids false results from interference with the event; however, in extreme cases, a positive test separated many years from a clinical manifestation also risks misclassification, as a causative relationship between event and test would then be in doubt. The Sapporo statement encouraged investigators to provide applicable information, but relevant existing data are rather poor. The stability of the laboratory testing over time is reassuring [24], yet spontaneous variation of aPL in individual patients occurs in up to a quarter of cases (Evidence Level II). Whether disease activity and treatment contribute to assay variability is unknown [25–27]. The committee suggests that researchers should not classify APS if more than 5 years separate the clinical event and the positive laboratory test, and that an allowance of at least 12 weeks between symptom and test will assist assessment of the relationship between clinical manifestations and aPL (Table 2). These time limits are valid independently of which feature of APS (clinical or laboratory) occurs first.

Persistent positivity of laboratory tests is important; the Sapporo criteria suggested an interval of at least 6 weeks between the two positive tests. In fact, there are no data to validate this interval. There are concerns that transient presence of epiphenomenal aPL – not infrequent in clinical practice – could risk misclassification (Evidence Level II) [28]. This committee proposes that increasing the interval to 12 weeks is

Table 2 Revized classification criteria for the antiphospholipid syndrome

Antiphospholipid antibody syndrome (APS) is present if at least one of the clinical criteria and one of the laboratory criteria that follow are met*

Clinical criteria

1. Vascular thrombosis†

One or more clinical episodes‡ of arterial, venous, or small vessel thrombosis§, in any tissue or organ. Thrombosis must be confirmed by objective validated criteria (i.e. unequivocal findings of appropriate imaging studies or histopathology). For histopathologic confirmation, thrombosis should be present without significant evidence of inflammation in the vessel wall.

2. Pregnancy morbidity

(a) One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, with normal fetal morphology documented by ultrasound or by direct examination of the fetus, or

(b) One or more premature births of a morphologically normal neonate before the 34th week of gestation because of: (i) eclampsia or severe pre-eclampsia defined according to standard definitions [11], or (ii) recognized features of placental insufficiency¶, or

(c) Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded.

In studies of populations of patients who have more than one type of pregnancy morbidity, investigators are strongly encouraged to stratify groups of subjects according to a, b, or c above.

Laboratory criteria**

1. Lupus anticoagulant (LA) present in plasma, on two or more occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Haemostasis (Scientific Subcommittee on LAs/phospholipid-dependent antibodies) [82,83].

2. Anticardiolipin (aCL) antibody of IgG and/or IgM isotype in serum or plasma, present in medium or high titer (i.e. > 40 GPL or MPL, or > the 99th percentile), on two or more occasions, at least 12 weeks apart, measured by a standardized ELISA [100,129,130].

3. Anti-β_2 glycoprotein-I antibody of IgG and/or IgM isotype in serum or plasma (in titer > the 99th percentile), present on two or more occasions, at least 12 weeks apart, measured by a standardized ELISA, according to recommended procedures [112].

*Classification of APS should be avoided if less than 12 weeks or more than 5 years separate the positive aPL test and the clinical manifestation. †Coexisting inherited or acquired factors for thrombosis are not reasons for excluding patients from APS trials. However, two subgroups of APS patients should be recognized, according to: (a) the presence, and (b) the absence of additional risk factors for thrombosis. Indicative (but not exhaustive) such cases include: age (> 55 in men, and > 65 in women), and the presence of any of the established risk factors for cardiovascular disease (hypertension, diabetes mellitus, elevated LDL or low HDL cholesterol, cigarette smoking, family history of premature cardiovascular disease, body mass index ≥30 kg m^{-2}, microalbuminuria, estimated GFR < 60 mL min^{-1}), inherited thrombophilias, oral contraceptives, nephrotic syndrome, malignancy, immobilization, and surgery. Thus, patients who fulfil criteria should be stratified according to contributing causes of thrombosis. ‡A thrombotic episode in the past could be considered as a clinical criterion, provided that thrombosis is proved by appropriate diagnostic means and that no alternative diagnosis or cause of thrombosis is found. §Superficial venous thrombosis is not included in the clinical criteria. ¶Generally accepted features of placental insufficiency include: (i) abnormal or non-reassuring fetal surveillance test(s), e.g. a non-reactive non-stress test, suggestive of fetal hypoxemia, (ii) abnormal Doppler flow velocimetry waveform analysis suggestive of fetal hypoxemia, e.g. absent end-diastolic flow in the umbilical artery, (iii) oligohydramnios, e.g. an amniotic fluid index of 5 cm or less, or (iv) a postnatal birth weight less than the 10th percentile for the gestational age. **Investigators are strongly advised to classify APS patients in studies into one of the following categories: I, more than one laboratory criteria present (any combination); IIa, LA present alone; IIb, aCL antibody present alone; IIc, anti-β_2 glycoprotein-I antibody present alone.

unlikely to affect sensitivity (Table 2); coupled with the prior proposal of an analogous interval between clinical manifestation and assay performance, it provides greater reassurance that the aPL detected are relevant to a predisposition to APS. We emphasize that proposed time intervals are based on expert opinion. Studies validating these time frames are imperative.

The committee advises against using the term 'secondary' APS. We could not find differences in the clinical consequences of aPL among patients in these two categories (Evidence Level I) [13,29]. Most patients with so-called secondary APS have SLE. It is unknown if APS and SLE are two diseases coinciding in an individual, if underlying SLE offers a setting for the development of APS, or if APS and SLE represent two elements of the same process [30,31]. Some cases with 'secondary' APS are classified as LLD. The interface between SLE, LLD and APS merits further consideration. Rather than distinguishing between patients with 'primary' and 'secondary' APS, documenting the coexistence of SLE (or other disease) is more advantageous for classification.

Finally, the entity of the Catastrophic Antiphospholipid Syndrome was outside the agenda of this workshop; a relevant consensus statement has been released [32].

Features associated with APS, but not included in the revised criteria

This panel also discussed clinical and laboratory features not included in the revised classification criteria for APS. These include: (i) heart valve disease, (ii) livedo reticularis (LR), (iii) thrombocytopenia, (iv) nephropathy, (v) neurological manifestations, (vi) IgA aCL, (vii) IgA anti-β_2GPI, (viii) antiphosphatidylserine antibodies (aPS), (ix) antiphosphatidylethanolamine (aPE) antibodies, (x) antibodies against prothrombin alone (aPT-A), and (xi) antibodies to the phosphatidylserine–prothrombin (aPS/PT) complex. Some of the features above are undoubtedly frequent but not specific in patients with APS. The committee considered that adoption of these features as independent criteria for definite APS may decrease diagnostic specificity, even though their association with APS is recognized.

Another issue is how to classify (i) cases with aPL and non-criteria clinical manifestations of APS, and (ii) the infrequent cases that fulfil the clinical criteria, but test positive only for non-criteria aPL. Some members of the committee proposed the term 'probable APS'. This concept was not

Table 3 Definition of aPL-associated cardiac valve disease

aPL-associated cardiac valve disease is:
Coexistence of aPL (Laboratory Criteria for APS) *along with*
Echocardiographic detection of lesions *and/or*
Regurgitation* *and/or* stenosis of mitral *and/or* aortic valve or any combination of the above.
Valve examination can be performed with TTE and/or with TEE
Defining valve lesions include:
Valve thickness >3 mm,
Localized thickening involving the leaflet's proximal or middle portion,
Irregular nodules on the atrial face of the edge of the mitral valve, *and/or* the vascular face of the aortic valve.
The presence and severity of regurgitation and/or stenosis should be documented with Doppler echocardiography.
Interpretation should be carried out by two expert echocardiographers.
Both functional capacity and objective assessment of heart status should be reported according to the revized NYHA Criteria for Diagnosis of Heart Disease [131].
Confirmation of valve disease may also be provided by histopathological findings of Libman-Sacks endocarditis in patients with concomitant SLE [132].
In all the above cases, the presence or history of rheumatic fever and infective endocarditis must be excluded.
Patients who fulfill Clinical Criteria for APS are excluded from the definition above.
Researchers should also state if the patient meets the American College of Rheumatology (ACR) revized criteria for SLE [133,134].

*Investigators are advised to consider moderate-severe mitral valve regurgitation as criterion for aPL-associated cardiac valve disease, as mild regurgitation is very common in the general population.

adopted, because the features listed above cannot be used as alternative criteria for APS. With these limitations in mind, we believe it would be reasonable to use these features, which were not selected for diagnosis of individual patients as 'probable APS', 'features associated with APS' or 'non-criteria features of APS'. For clinical studies, patients falling into any of these categories should be classified separately from those that fulfill the revised classification criteria for APS. This policy may help clarify unsettled issues (specificity, associations of aPL with clinical manifestations, and differences in outcome and impact of treatment) between those features and definite APS. Thus, this committee encourages the separate recognition of non-criteria features of APS, and proposes a terminology (Tables 3–6). The evidence that precludes adoption as criteria is summarized in the section on Specific issues.

Specific issues

Cardiac manifestations

Heart valve lesions (vegetations, valve thickening and dysfunction) are frequent in APS, independent of SLE [33], but data are contradictory because of differences in echocardiography technique and descriptions for findings, inconsistent associations with aPL, and population heterogeneity[1] (Evidence Level II) [36,37]; confounding factors associated with cardiac valve disease include age, hypertension and obesity (Evidence Level I) [38]. The committee proposes a minimal consensus regarding the valve dysfunction and provides relevant definitions of heart valve lesions in APS (Table 3), but recommends against adoption as criteria. Determination of aPL in patients coming to medical attention because of valve disease should be individualized rather than routine.

Coronary artery disease (CAD) fulfills the thrombosis criterion for APS; we recommend that patients be stratified according to thrombosis risk stratification guidelines (Table 2). The workshop advises against routine performance of aPL tests in patients with CAD unless the patient's young age and lack of identifiable risk factors suggest a rare etiology.

Few data exist concerning the incidence of ventricular dysfunction in APS (Evidence Level IV). The committee advises that the rare cases with biopsy-proven myocardial microthrombosis, or with intracardiac thrombi be recognized as meeting the thrombosis criterion for APS (Evidence Level IV). Detection of cardiac microthrombosis or intracardiac thrombi without apparent explanation warrants aPL testing.

Neurological manifestations

Transient cerebral ischemia and stroke fall within the spectrum of thrombosis; thus, pertinent stratification recommendations apply. A consensus report on these manifestations has been published [39].

Antiphospholipid antibodies correlate with physical disability in the elderly [40] (Evidence Level II). In one small study of APS patients without SLE [41], long-term presence of LA is a risk factor for dementia (Evidence Level II). In SLE patients, persistent elevation of aPL is associated with cognitive dysfunction (Evidence Level I) [42,43]. Prospective studies deny an association between migraine and aPL (Evidence Level I) [44,45]. In patients with multiple sclerosis (MS), an association between aPL and clinical course cannot be supported (Evidence Level I) [46]. Patients with concomitant MS and SLE may be an exception, but studies are contradictory (Evidence Level II) [47,48]. Transverse myelopathy (TM) is a rare entity within APS [33]. Limited data suggest that in the 1% of SLE patients who manifest TM, the latter is associated with aPL (Evidence Level IV) [49]. Contradictory data exist on the relationship between aPL and seizures in SLE (Evidence Level I) [50,51] and in epilepsy patients (Evidence Level II) [52,53]. In unselected APS patients, epilepsy has been retrospectively associated with SLE, CNS ischemic events, thrombocytopenia, and LR (Evidence Level II) [54]. It is uncertain whether aPL can influence the clinical course of epilepsy, as relevant prospective data are missing. This committee considers that

[1]For instance, although mitral valve thickness >3 mm, measured with TEE, correlated significantly with aCL >40 GPL in one study [34], the average mitral valve thickness in the control population of another study was 3.2 mm with Doppler echocardiography [35].

Table 4 Definition of aPL-associated livedo reticularis (LR)

aPL-associated LR is the coexistence of aPL (Laboratory Criteria for APS) and LR.

Livedo reticularis is the persistent, not reversible with rewarming, violaceous, red or blue, reticular or mottled, pattern of the skin of trunk, arms or legs. It may consist of regular unbroken circles (regular LR) or irregular-broken circles (livedo racemosa). The width of the branching pattern can be ≥10 mm (large LR) or < 10 mm (fine LR). Four variants may be recognized: fine livedo racemosa, large livedo racemosa, fine regular LR, and large regular LR.

Pathologic changes confirmative, but not required, for LR classification and diagnosis include partial or complete occlusion of the lumen of small- to medium-sized arteries and/or arterioles at the dermis-subcutis border with no evidence of perivascular inflammatory infiltrate and negative direct immunofluorescence examination [62].

Patients who fulfill Clinical Criteria for APS are excluded from the definition above.

Table 5 Definition of aPL-associated nephropathy (APLN)

aPL-associated nephropathy [66,68] is the coexistence of aPL (Laboratory Criteria for APS) along with the histopathologic detection of:

Thrombotic microangiopathy involving both arterioles and glomerular capillaries *and/or*

One or more of:

- Fibrous intimal hyperplasia involving organized thrombi with or without recanalization
- Fibrous and/or fibrocellular occlusions of arteries and arterioles
- Focal cortical atrophy
- Tubular thyroidization (large zones of atrophic tubules containing eosinophilic casts)

Vasculitis, thrombotic thrombocytopenic purpura, hemolytic uremic syndrome, malignant hypertension, and other reasons for chronic renal ischemia are exclusions.

Patients who fulfill Clinical Criteria for APS are excluded from the definition above

If SLE is also present, the above lesions should be distinguished from those associated with lupus nephropathy.

Table 6 Definition of aPL-associated thrombocytopenia

aPL-associated thrombocytopenia is the coexistence of aPL (Laboratory Criteria for APS) *along with* the following:

Thrombocytopenia ($< 100 \times 10^9$ L^{-1}), confirmed at least twice 12 weeks apart.

Exclusion of patients with thrombotic thrombocytopenic purpura, disseminated intravascular coagulation, pseudo-thrombocytopenia, and heparin-induced thrombocytopenia [135,136].

Thrombocytopenia is further characterized as moderate (platelet count $50–100 \times 10^9$ L^{-1}) or severe ($< 50 \times 10^9$ L^{-1}).

Subclassification of patients according to the presence or absence of SLE is advantageous.

Patients who fulfill Clinical Criteria for APS are excluded from the definition above.

there is insufficient evidence to include cognitive dysfunction, headache or migraine, MS, TM, and epilepsy in the revized APS classification criteria; however, the data concerning cognitive dysfunction are suggestive and warrant further study.

Skin manifestations

Livedo reticularis is more prevalent among APS patients with SLE, and in females (Evidence Level II) [33,55]. Studies of associations with specific aCL isotypes or LA are contradictory (Evidence Level II) [56–60]. In unselected APS patients, LR has been retrospectively correlated with aCL and arterial thrombosis, but not with anti-β_2GPI or LA, venous thrombosis, or pregnancy morbidity (Evidence Level II) [61]. There are no prospective studies on the ability of LR to predict thrombosis, with the exception of the rare Sneddon's syndrome (Evidence Level II) [62]. The LR lesions can lead to ischemia and tissue infarction, called livedo vasculitis (purpuric macules, cutaneous nodules, and/or painful ulcerations) [63]; stating its presence is advisable. Inclusion of LR as an independent clinical criterion for APS would not serve to classify homogeneous patient groups, and definition is required (Table 4). The committee advises subclassification of LR variants for clinical studies. Although histologic findings sometimes may be helpful in most LR cases, there are no pathognomonic findings [62]; performing a biopsy is not routinely indicated or encouraged by this committee.

Other skin manifestations of APS include skin ulcerations, pseudo-vasculitic lesions, digital gangrene, superficial phlebitis, malignant atrophic papulosis-like lesions, subungual splinter hemorrhages [63], and anetoderma (a circumscribed area loss of dermal elastic tissue) (Evidence Level IV) [64]. They are rare, and none merits inclusion as a criterion.

Renal manifestations

Antiphospholipid antibodies correlate with lesions of renal small-artery vasculopathy and chronic renal ischemia (Evidence Level III) [65–68]. The committee recommends the term 'aPL-associated nephropathy' (APLN) to describe this entity (Table 5). Renal lesions are identical in SLE–APS and non-SLE–APS patients, and have been associated with extra-renal vascular thrombosis and pregnancy complications in SLE patients (Evidence Level II) [65,66,69,70]. They are independent of lupus nephritis and do not correlate with the rate of loss of renal function or end-stage renal disease [65,66]. Apart from thrombotic microangiopathy, which represents an acute event, other lesions of APLN reflect chronic vascular damage, are more frequent [66,68], and may be non-specific. In almost all reported cases, the diagnosis of APLN derived from multiple findings. Histologic criteria for APLN have not been validated. Patients with histologically proven APLN satisfy the thrombosis criterion for APS, provided that other conditions resulting in similar renal lesions are excluded. This committee does not suggest routine performance of renal biopsy in APS: this decision should be guided by conventional clinical indications.

Thrombocytopenia

Antiphospholipid antibodies are frequently found in patients initially diagnosed with idiopathic thrombocytopenic purpura (ITP), prospectively associated with thrombosis (Evidence Level I) [71,72]. This may suggest that aPL confers a high risk of thrombosis in patients with ITP, or that ITP is a first

symptom of APS, as it may be of SLE. Thrombocytopenia is more common in patients with APS and SLE than in patients with APS alone (Evidence Level II) [33]. Antibodies directed against platelet glycoproteins are associated with thrombocytopenia (but not with thrombosis) in patients with aPL [73], and also in patients with APS, comparable with ITP patients [74] (Evidence Level III). The committee consented that thrombocytopenia occurring in patients with persistent aPL, in the absence of clinical manifestations of APS, should be considered to be different from ITP: such patients have an increased thrombotic risk and require closer follow-up. On the other hand, inclusion of thrombocytopenia as an independent clinical criterion for APS would likely add little to sensitivity with a potential cost in specificity; a clear distinction from thrombocytopenia because of SLE and ITP are required, and relevant data from prospective studies are inadequate. We propose the term 'aPL-associated thrombocytopenia' (Table 6) to stratify patients for clinical studies. We propose a platelet count $< 100 \times 10^9\ L^{-1}$ as the upper cut-off limit for thrombocytopenia in APS (Evidence Level II) [33]. This relatively stringent limit (vs. $150 \times 10^9\ L^{-1}$) could serve the criteria target of maximum specificity, while including the severe and moderate cases.

Lupus anticoagulant

Lupus anticoagulant better correlates with thrombosis (Evidence Level I) [75], pregnancy morbidity (Evidence Level II) [76], and thrombosis in SLE patients (Evidence Level I) [77] than does aCL. Inter-laboratory agreement is relatively poor for the large number of LA assays on the market [78,79]. The present committee recommends that laboratories performing LA comply with existing rules to improve inter-laboratory concordance (Evidence Level II) [78,80–83].

No definite recommendation can be given on the assays of choice for LA testing. Both activated partial thromboplastin time (APTT)-based assays and dilute Russell's viper venom time (dRVVT) are suitable for LA (Evidence Level II) [79,80], provided that the APTT used for LA testing is LA sensitive. One positive test suffices for LA positivity; as no single test is 100% sensitive for LA, it is advised to use two or more tests with different assay principles before the presence of LA is excluded.

Unless one uses an LA test system that includes a heparin neutralizer (most of the commercial dRVVT-assays), the thrombin time should always be measured to exclude unforeseen presence of unfractioned heparin. If the patient is on oral anticoagulants, measurement of LA is better postponed (Evidence Level III) [84], or patient samples be diluted 1 : 2 with normal plasma before the test is performed, provided that international normalized ratio (INR) is < 3.5. When INR is > 3.5, the LA testing is unworkable (Evidence Level IV). Several phospholipids (rabbit brain extract, hexagonal phase phospholipids, defined phospholipid vesicles, washed-activated platelets, frozen-thawed platelets and lyophilized platelet extracts) have been used successfully in LA confirmation assays; no evidence exists for superiority of any particular one.

Little objective information and no relevant guidelines exist to define a positive-screening test [82]. The use of fixed-time cut-off limits in different laboratories with different instruments was discouraged by this panel. For dRVTT and other LA assays, better precision can be achieved when individual laboratories determine their own cut-off levels for positive results (Evidence Level II) [78,80,85]. Use of normalization ratios (test sample : control sample) is the best way to compensate for inter- and intra-assay variation. Clotting time ratios (test/control) of > 1.1 for the dRVTT and > 1.2 for KCT are applied in many laboratories, and indicate LA according to earlier guidelines [86,87]. Ratios have high specificity but may have low sensitivity (Evidence Level II) [80]. An appropriate way to establish cut-off levels is to measure LA in 40 healthy controls and determine the geometric mean + 2SD.

The interpretation of the confirmation procedure – the so-called lupus ratio (mathematically identical with the screen/confirm ratio), or the confirm/normal ratio – that offers the highest risk for thromboembolic complications remains uncertain. Integration of screening and confirmation into a single assay makes LA testing less time consuming, and may increase diagnostic accuracy and inter-laboratory agreement (Evidence Level III) [88]. Nevertheless, the result of the LA test that best correlates with clinical events of APS cannot be concluded. Until further evidence is provided, the committee advises considering positive every sample outside the normal range (Evidence Level IV).

The use of widely available pooled patient plasmas for positive controls (instead of the traditional plasma samples of local LA-positive patients) is currently advised [83]. Plasma spiked with monoclonal antibodies will soon be available; its use and standardization through participation in multicenter studies (e.g. ECAT and Scientific and Standardization Committee [SSC]) are encouraged.

Two new methods enable discrimination between β_2GPI and prothrombin antibodies causing LA. The first uses cardiolipin vesicles and can only be used in an APTT-based assay [89]. The second is based on changes in the final calcium concentration in the assay, but does not work when a mixture of both anti-β_2GPI and antiprothrombin antibodies is present [90]. In patients with autoimmune diseases, β_2GPI-dependent LA strongly correlates with a history of thrombosis, in contrast to the β_2GPI-independent assay (Level of Evidence II) [91]. An international multicenter trial to confirm this assertion will begin shortly.

Anticardiolipin assay

Interlaboratory agreement on aCL measurement remains marginal with both home-based and commercial assays (Evidence Level I) [14,92,93]. Discrepancies are mainly because of cut-off, calibration, and other methodological issues. Expression of aCL assays in ranges of positivity achieves better interlaboratory and inter-run agreement than do quantitative readouts [94]. We note that IgM aCL tends to give false-positive results, particularly in the low-positive