of atorvastatin on serum lipids, lipoproteins, and hemostasis. Am J Hematol, 78(1):1-6, 2005;

・Kamikura Y, Wada H, Nobori T, Matsumoto T, Shiku H, Ishikura K, Yamada N, Nakano T, Kazahaya Y, Sawai T, Matsuda M: Elevated plasma levels of fibrin degradation products by granulocyte- derived elastase in patients with deep vein thrombosis. Thromb Res, 115(1): 53-57, 2005

別添６

研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表 (H16 年度)

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kobayashi,H.,Hosono,O.,Iwata,S.,Kawasaki,H.,Kuwana,M.,Tanaka,H.,Dang,N.H.,Morimoto,C.	The tetraspanin CD9 is preferentially expressed on the human $CD4^+CD45RA^+$ naïve T cell population and is involved in T cell activation.	Clin Exp Immunol	137 : 1	101-108	2004
Kuwana,M.,Okazaki,Y.,Yasuoka,H.,Kawakami,Y,Ikeda,Y.	Defective vasculogenesis in systemic sclerosis.	Lancet	364: 9434	603-610	2004
Kuwana,M.	β_2-glycoprotein I: antiphospholipid syndrome and T-cell reactivity.	Thromb Res	114 : 5-6	347-355	2004
Yasuoka,H.,Okazaki,Y.,Kawakami,Y.,Hirakata,M.,Inoko,H.,Ikeda,Y., Kuwana,M.	Autoreactive $CD8^+$ cytotoxic T lymphocytes to major histocompatibility complex class I chain-related molecule A in patients with Behçet's disease.	Arthritis Rheum	50 : 11	3658-3662	2004
Satoh,T.,Kimura,K.,Okano,Y.,Hirakata ,M.,Kawakami,Y.,Kuwana,M.	Lack of circulating autoantibodies to bone morphogenetic protein receptor-II or activin receptor-like kinase 1 in mixed connective tissue disease patients with pulmonary arterial hypertension.	Rheumatology	44 : 2	192-196	2005
Kuwana,M.,Matsuura,E.,Kobayashi,K.,Okazaki,Y.,Kaburaki,J.,Ikeda,Y,Kawakami,Y.	Binding of β_2-glycoprotein I to anionic phospholipids facilitates processing and presentation of a cryptic epitope that activates pathogenic autoreactive T cells.	Blood	105 : 4	1552-1557	2005
Fujimura,K.,Kuwana,M.,Kurata,Y.,Imamura,M.,Harada,H.,Sakamaki,H.,Teramura,M.,Koda,K.,Nomura,S.,Sugihara,S.,Shimomura,T.,Fujimoto,T.,Oyashiki,K.,Ikeda,Y.	Is eradication therapy useful as the first line of treatment in *Helicobacter pylori*-positive idiopathic thrombocytopenic purpura? Analysis of 207 eradicated chronic ITP cases in Japan.	Int J Haematol	81 : 2	162-168	2005
Kuwana,M.,Ikeda,Y.	The role of autoreactive T-cells in the pathogenesis of ITP.	Int J Haematol	81 : 2	106-112	2005
Kokame K & Miyata T	Genetic defects leading to hereditary Thrombotic Thrombocytopenic Purpura	Semin Hematol	41	334-340	2004
Bannno F, Kaminaka K, Soejima K, Kokame K & Miyata T	Identification of strain-specific variants of mouse ADAMTS13 gene encoding von Willerand Factor-cleaving protease	J of Biol Chem	279:29	30896-60903	2004
Ishikuara K, Wada H, Kamikura Y, Hattori K, Fukuzawa T, Yamada N, Nakamura M, Nobori T & Nakano T	High prevalence of anti-prothrombin antibody in patients with deep vein thrombosis	Am J of Hematol	76	338-342	2004
Matsumoto M, Yagi H, Ihizashi H, Wada H & Fujimura Y	The Japanese experience with Thromboic Thrombocytopenic Purpura- Hemolytic uremic syndrome	Semin Hematol	41	68-74	2004
Kawahara M, Kannno M, Matsumoto M, Nakamura S, Fujimura Y & Ueno S	Diffuse neurodeficits in intravascural lymphomatosis with ADAMTS13 inhibitor	Eurology	63	1731-1733	2004
Kamikura Y, Wada H, Nobori T, Matsumoto T, Shiku H, Ishikura K, Yamada N, Nakano T, Kazahaya Y, SawaiT & Matsuda M	Elevated plasma levels of fibrin degradation products by granulocyte-derived elastase in patients with deep vein thrombosis	Thromb Res	115	53-57	2005
Kushiya F, Wada H, Ooi K, Sakurai Y, SAkaguchi A, Noda M, Abe Y Nakasaki T, Tsukada T, Shiku H & Nobori T	Effects of atorvastatin on serum lipids, lipoproteins and hemostasis	Am J of Hematol	78	1-6	2005
Furukoji E, Matsumoto M, Yamashita A, Yagi H, Sakurai Y, Marutsuka K, Hatakeyama K, Morishita K, Fujimura Y, Tamura S & Asada Y	Adenovirus-mediated transfer of human placental ectonucleoside triphosphate diphosphohydrolase to vascular smooth muscle cells suppresses platelet aggregation in vitro and arterial thrombus formatin in vivo	Circulation	111	808-815	2005

研究成果の刊行物・別冊

Clin Exp Immunol 2004; 137:101–108 doi:10.1111/j.1365-2249.2004.02494.x

The tetraspanin CD9 is preferentially expressed on the human CD4$^+$CD45RA$^+$ naive T cell population and is involved in T cell activation

H. KOBAYASHI*, O. HOSONO*, S. IWATA*, H. KAWASAKI*, M. KUWANA†, H. TANAKA*, N. H. DANG‡ & C. MORIMOTO*‡ *Division of Clinical Immunology, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan, †Division of Cellular Signalling, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan, and ‡Department of Lymphoma/Myeloma, MD Anderson Cancer Center, Houston, TX, USA

(Accepted for publication 31 March 2004)

SUMMARY

Human CD4$^+$ T cells can be divided into reciprocal memory and naive T cell subsets based on their expression of CD45 isoforms and CD29/integrin beta1 subunit. To identify unique cell surface molecules on human T cells, we developed a new monoclonal antibody termed anti5H9. Binding of anti5H9 triggers a co-stimulatory response in human peripheral blood T cells. Retrovirus-mediated expression cloning has revealed that the antigen recognized by anti5H9 is identical to the tetraspanin CD9. We now show that human CD9 is preferentially expressed on the CD4$^+$CD45RA$^+$ naive T cell subset, and that CD9$^+$CD45RA$^+$ T cells respond preferentially to the recombinant beta$_2$-glycoprotein I, compared to CD9$^-$CD45RA$^+$ T cells. Furthermore, anti5H9 inhibits both the recombinant beta$_2$-glycoprotein I- and the recall antigen tetanus toxoid-specific T cell proliferation. These results suggest that the tetraspanin CD9 plays an important role in T cell activation.

Keywords beta$_2$-glycoprotein I CD9 CD45RA T cell tetraspanin

INTRODUCTION

Human CD4$^+$ T cell population is a heterogeneous collection of lymphocytes having different phenotypic and functional properties. These cells can be divided into functionally distinct and largely reciprocal subsets based on their differential expression of CD45 isoforms (CD45RA and CD45RO) and CD29/integrin beta1 subunit [1–8]. The CD4$^+$CD45RO$^+$CD29^{high} memory (helper inducer) subset responds preferentially to soluble recall antigens such as tetanus toxoid (TT), and provides a strong helper function for IgG production by B cells [1,3]. In contrast, the CD4$^+$CD45RA$^+$CD29^{low} naive (suppressor inducer) subset responds poorly to recall antigens and lacks the helper function, but this T cell subset proliferates maximally in autologous mixed lymphocyte reaction (AMLR) [2]. Among the many hypotheses regarding the biological significance of AMLR *in vivo*, it has been suggested that AMLR reflects a self-recognition process in the normal immune response [9,10]. In addition, it has been reported recently that CD4$^+$CD45RA$^+$ T cells proliferate in response to self-antigens *in vitro*, such as heat shock protein 60 or myelin basic protein [11,12]. These results suggest that human CD4$^+$CD45RA$^+$ T cells include autoreactive T cells, and it is conceivable that these cells might be involved in autoimmunity.

Many cell surface molecules have been defined and characterized by the use of specific monoclonal antibodies (MoAbs). Although many cell surface molecules preferentially expressed on CD4$^+$CD45RO$^+$ memory T cells have been identified, such as LFA-1, CD2 [13], CD29 [1], CD26 [14], CD82 [15] and CD43 [16], only a few molecules expressed preferentially on CD4$^+$CD45RA$^+$ naive T cells have been characterized thus far, including CD27 [17], CD31 [18] and CD62L [19]. In addition, in contrast to CD4$^+$CD45RO$^+$ T cells, the function of CD4$^+$CD45RA$^+$ T cells remains poorly understood.

CD9 belongs to the tetraspanin family, which consist of CD37, CD53, CD63, CD81, CD82, CD151, and a growing number of new proteins such as Tspan-1–6 and RDS/peripherin [20–22]. The tetraspanins are characterized by the presence of four conserved transmembrane regions [20–22] and are implicated in the regulation of cell–cell adhesion [23,24], cell motility [25–28], tumour cell metastasis [25–28], cell fusion [29–31], signal transduction [15,32] and cellular activation [15,32,33]. Tetraspanin is currently viewed to function as the organizer, facilitator or adaptor which assembles various molecular complexes on cell surfaces and participates in the signalling activity with associated molecules [22].

Recent gene targeting technology provides additional insight into the function of tetraspanin *in vivo*. Altered immune

Correspondence: Chikao Morimoto MD, Division of Clinical Immunology, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108–8639, Japan.

E-mail: morimoto@ims.u-tokyo.ac.jp

response has been reported in mice lacking CD81 or CD37 [34,35]. These results suggest that tetraspanins are involved in the regulation of the immune system. However, alterations in the immune system have not yet been observed in CD9-deficient mice, which showed reduced fertility [29–31]. In the murine system, it has been shown that CD9 is an inducer of co-stimulation and activation-induced cell death on T cells [32,33,36,37]. Meanwhile, CD9 function and distribution in human T cells remain largely unknown.

In the present study, we developed a new MoAb termed anti5H9 that reacts with human CD9, a member of the tetraspanin superfamily. Furthermore, CD9 is preferentially expressed on naive T cell population and anti5H9 inhibits both the recombinant beta$_2$-glycoprotein I- and the recall antigen tetanus toxoid-specific T cell proliferation. These findings suggest that CD9 may play a role in the process of T cell activation.

MATERIALS AND METHODS

Antibodies

The MoAb anti5H9 was established by fusing a myeloma cell line with spleen cells of BALB/c J mice after immunization with the erythroleukaemia cell line K562 and developed hybridoma producing anti5H9 by limiting dilution method [1,2]. The isotype of anti5H9 was determined to be IgG_1 kappa by the mouse MoAb isotyping kit (Amersham Biosciences, UK). The MoAbs, anti-CD3 (OKT3), anti-CD29 (4B4) [1], anti-CD45RA (2H4) [2], anti-CD45RO (UCHL-1) [3], anti-HLA-DR (L234), anti-CD82 (4F9) [15] and anti-CD28 (4B10) [1] were all purified from ascites fluid. The labelled MoAbs used were: fluorescein isothiocyanate (FITC)-conjugated anti-CD45RA (HI100, PharMingen, San Diego, CA, USA), RD1-conjugated anti-CD3 (UCHT1, Beckman Coulter, Inc., Fullerton, CA, USA), phycoerythrin (PE)-conjugated anti-CD4 (RPA-T4, PharMingen), anti-CD8 (RPA-T8, PharMingen), anti-CD45RA (2H4, Beckman Coulter), anti-CD45RO (UCHL-1, PharMingen), anti-CD62L (SK11, Immunocytometry Systems, San Jose, CA, USA), anti-CD29 (4B4, Beckman Coulter), PerCP-conjugated anti-CD4 (SK3, Immunocytometry Systems), anti-CD8 (SK1) (Immunocytometry Systems). FITC-, PE- and PerCP-conjugated control mouse IgG_1, anti-CD11a and streptavidin–allophycocyanin (APC) were obtained from PharMingen. Biotinylation or FITC-conjugation of anti5H9 was performed as described previously [16]. FITC-conjugated goat antimouse Ig and control mouse IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

Preparation of cells

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density-gradient centrifugation. Unfractionated mononuclear cells were depleted of monocytes by adherence to plastic dishes. T cells (>95% $CD3^+$) were then purified by E rosetting [1,2]. Further removal of monocytes from T cells was achieved by incubation with 5 mM L-leucine methyl ester HCl (Sigma) for co-stimulation assay. $CD45RA^+$ T cells and $CD45RO^+$ T cells were obtained from E rosette-positive cells by negative selection with anti-CD45RA (2H4), anti-CD45RO (UCHL-1) and goat antimouse IgG-conjugated immunomagnetic beads (PerSeptive Biosystems, Framingham, MA, USA) [15].

Cell lines

T cell lines (HPB-ALL and H9) and the erythroleukaemia cell line (K562) used in this study were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. An ecotropic retrovirus packaging cell line, BOSC23 (ATCC CRL 11554) was maintained in Dulbecco's modified Eagle medium (DMEM), containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 15 μg/ml hypoxanthine, 2 μg/ml aminopterin, 6 μg/ml thymidine, 250 μg/ml xanthine and 25 μg/ml mycophenolic acid. An amphotropic retrovirus packaging cell line, Phoenix (kindly provided by Dr Nolan) was maintained in DMEM, containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. A murine pre-B cell line Ba/F3 has been described previously [38,39].

Analysis and separation of cells by flow cytometry

Flow cytometric analysis was performed on an Epics XL cell sorter (Coulter Electronics, Hialeah, FL, USA) with System II software or FACSCalibur (Becton Dickinson, Franklin Lake, NJ, USA) as described previously [15]. For all samples, lymphocytes were analysed by selective gating based on the parameters of forward and side scatter. Cell sorting was fulfilled on an Epics Elite cell sorter (Coulter Electronics). In all experiments, post-sorting viability was more than 95% by Trypan blue dye exclusion. Purity of separated T cell subsets was always more than 95%.

Retrovirus-mediated expression cloning

Oligo (dT)-cDNA libraries were constructed from HPB-ALL cells, which steadily express 5H9 antigen [38,39]. Recombinant retroviruses containing the cDNA library were produced and used for infection [38,39]. In brief, packaging cell line BOSC23 cells were transfected with cDNA derived from HPB-ALL cells by Lipofectamine reagent (GIBCO BRL, Grand Island, NY, USA). After 2 days, the culture supernatant containing recombinant retroviruses was harvested and used for infection of Ba/F3 cells. At 48 h after infection, the cells were subjected to flow cytometric analysis. After three cycles of sorting of the cells reactive with anti5H9, 5H9 antigen-positive cells were cloned. Genomic DNA was isolated from each clone and the integrated cDNA segment was amplified by polymerase chain reaction (PCR) using primers to retroviral vector pMX [40]. The PCR reaction was performed for 38 cycles (30 s at 96°C, 30 s at 58°C and 4 min at 72°C) with the LA PCR kit version 2 (Takara Shuzo Co. Ltd, Tokyo, Japan). The resulting PCR fragments were subcloned into the TA cloning vector pCR2·1 (Invitrogen) and sequenced using the PRISM dye terminator cycle sequencing ready reaction kit (Applied Biosystems, UK) and the ABI 373S sequencing system. All sequences of the cDNA were determined by constructing deletion mutant clones. Furthermore, the retroviral vector inserted the cloned cDNA was constructed again and transfected to H9 cells, which did not express the CD9 molecule, with amphotropic packaging cell line Phoenix cells in the same manner.

Western blotting

Cells were lysed with lysis buffer (25 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM phenylmethylsulfonylfluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 mM NaF, 1% CHAPS). The cell lysates were denatured with sample buffer with or without 5% 2-mercaptoethanol at 95°C for 5 min. The samples were subjected to SDS-PAGE and electrotransferred

onto PVDF membranes (Millipore, Bedford, MA, USA). The blots were blocked with 5% skimmed milk in Tris-buffer solution containing 0·05% Tween-20 (TBS-T) for 1 h at room temperature. The blots were washed three times with TBS-T and incubated for 1 h at room temperature in TBS-T with 1 μg/ml anti5H9. After washing three times, the blots were incubated with horseradish peroxidase-conjugated goat antimouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), and the bands were visualized according to the chemiluminescent reaction method (ECL Western blotting detection reagents, Amersham Biosciences). The blots were then exposed to X-ray film for a few minutes.

Co-stimulation assay

Monoclonal antibodies were immobilized to wells of 96-well flat-bottomed microculture plates (Corning Glass Works, Corning, NY, USA) at appropriate concentrations in a final volume of 0·1 ml. Purified T cells at $2{\cdot}0 \times 10^5$ cells/well were cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C in humidified atmosphere with 5% CO_2 for the indicated period. Proliferation of T cells was assessed by incorporation of [^{3}H]-thymidine in the last 13 h of cultures. The results were expressed as mean cpm ± s.d. of triplicate cultures.

T cell proliferation assay

Antigen-induced T cell proliferation was assayed by Hattori's methods with some modification [41]. Briefly, purified CD9⁺CD45RA⁺ T cells or CD9⁻CD45RA⁺ T cells were cultured with or without antigen in the presence of 6% of macrophages in 96-well round-bottomed culture plates for 7 days, and then proliferation was assessed by incorporation of [^{3}H]-thymidine in the last 13 h of cultures. Recombinant beta$_2$-glycoprotein I (GPI) (10 μg/ml) produced by *Escherichia coli* expression system or tetanus toxoid (TT, 5 ng/ml) (purchased from Calbiochem, CA, USA) was added to the cultures. In parallel, phytohaemagglutinin (5 μg/ml) was used to stimulate T cells to evaluate the non-specific responsiveness of T cells.

Statistics

Student's *t*-test or Welch's *t*-test was used to determine whether the difference between control and sample was statistically significant ($P < 0{\cdot}05$ being significant).

RESULTS

5H9 antigen is identical to a tetraspanin family molecule, human CD9

In an attempt to identify novel cell surface molecules on human T cells, we developed a variety of hybridoma clones and selected a clone producing a monoclonal antibody strongly reactive to human peripheral blood T cells, termed anti5H9 (5H9⁺CD3⁺ 26·6 ± 9·5%, $n = 5$) (Fig. 1a). To characterize the 5H9 antigen, we performed cDNA cloning of the 5H9 antigen from a cDNA library of HPB-ALL constitutively expressing the 5H9 antigen by the retrovirus-mediated expression cloning system, and then sequenced the isolated cDNA. As a result of a homology search on the DDBJ/EMBL/GenBank database, it was determined that the nucleotide sequence of cDNA encoding the 5H9 antigen is identical to that of human CD9, a member of the tetraspanin superfamily, containing 684 base pairs (bp) of open reading frame of CD9 cDNA [DDBJ databases; nucleotide sequence data of CD9 are available in the DDBJ databases under Accession number(s) AB079244]. The isolated cDNA was again inserted into the retrovirus vector (pMX-5H9-1) and the gene product of the cDNA was expressed on the CD9-negative T cell line H9 (H9–5H9-1). The reactivity of anti-CD9 MoAbs to this cell line was subsequently analysed by flow cytometry. As shown in Fig. 1(b), H9–5H9-1 cells bound specifically to both ALB-6 (a commercially available anti-CD9 MoAb) and anti5H9, whereas these MoAbs did not react with parent H9 cells. The irrelevant MoAb, anti-CD29 (4B4), reacted with H9–5H9-1 as well as parent H9 cells. Next, Western blot analysis of cell lysate derived from H9–5H9-1 cells showed that a single band with molecular size of 24 kDa was detected by anti5H9 under both reducing and non-reducing conditions (Fig. 1c). Furthermore, co-stimulation assays for anti5H9-mediated comitogenic response in human peripheral blood T cells showed that anti5H9 could induce a dose-dependent co-mitogenic effect with a submitogenic dose of anti-CD3, whereas anti5H9 alone did not induce T cell proliferation, as demonstrated by a representative experiment shown in Fig. 1d. Based on the above results, we concluded that the 5H9 antigen is identical to human CD9, a member of the tetraspanin superfamily, and that anti5H9 can provide co-stimulatory signal to human peripheral T cells.

CD9 is expressed preferentially on the human CD4⁺CD45RA⁺ T cell population

We next examined the expression of CD9 on subsets of human peripheral blood T cells by flow cytometric analysis using anti5H9. According to data from assays involving two-colour staining of human PBMC, the expression level of CD9 on T cells was relatively modest, with the population of CD9⁺ T cells being relatively small. CD9 was expressed on both CD4⁺ and CD8⁺ T cells (CD9⁺CD4⁺ 12·9 ± 9·0%, CD9⁺CD8⁺ 14·1 ± 2·4%, respectively). In addition, we performed four-colour staining of human PBMC. As shown by a representative staining pattern (Fig. 2a), CD9 was expressed preferentially on the CD4⁺CD45RA⁺ naive subset within human T cells (CD4⁺CD45RA⁺CD9⁺ 22·6 ± 10·2% *versus* CD4⁺CD45RO⁺CD9⁺ 4·9 ± 3·7%, $n = 5$, $P < 0{\cdot}05$). Moreover, as shown in Fig. 2b, almost all the CD4⁺CD45RA⁺CD9⁺ T cells express L-selectin (CD62L), which is the homing receptor and is also a marker for naive T cells, while displaying a low level of CD29 (data not shown). These results indicate that CD9 is expressed preferentially on the subset of human CD4⁺CD45RA⁺ naive T cells.

CD9⁺CD45RA⁺ T cells respond maximally to beta$_2$-GPI

We next focused on the property of CD9⁺ T cells within the CD4⁺CD45RA⁺ T cell subset. To address the issue, we assessed the difference in proliferative response between CD9⁺CD45RA⁺ and CD9⁻CD45RA⁺ cells, as it has been suggested that CD4⁺CD45RA⁺ T cells contain the responding population to autoantigens [11,12]. We first examined the proliferative response of CD45RA⁺ and CD45RO⁺ T cells to recombinant beta$_2$-GPI. Beta$_2$-GPI is the most common antigenic target molecule in the antiphospholipid syndrome (APS) and is regarded as an autoantigen. *In vitro*, it has been reported that human CD4⁺ T cells from healthy individuals as well as APS patients with HLA-DR53 respond to recombinant beta$_2$-GPI [41]. CD45RA⁺ T cells and CD45RO⁺ T cells were purified from

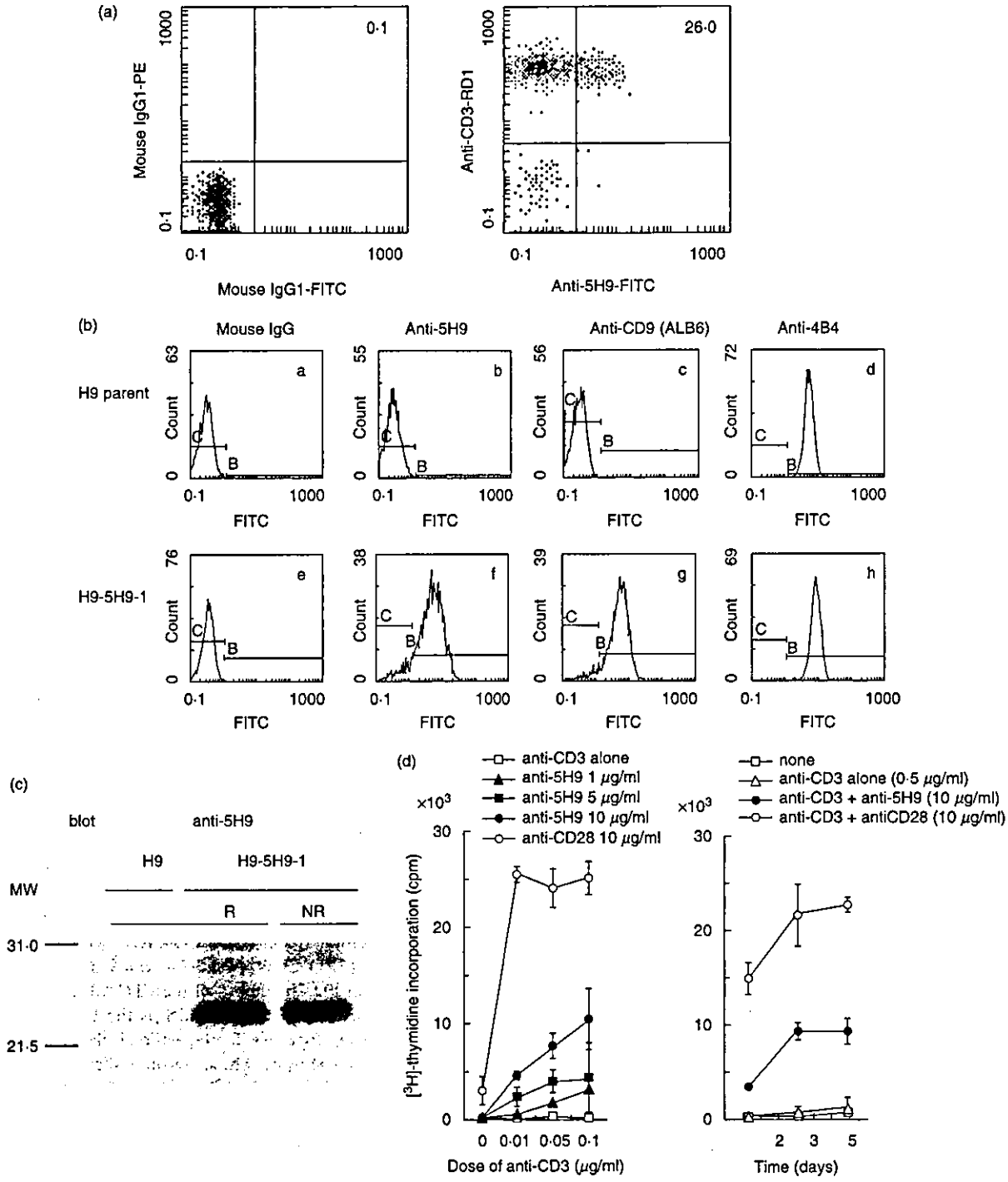

Fig. 1. 5H9 antigen is identified to be the CD9 molecule. (a) Reactivity of anti5H9 MoAb to human peripheral blood T cells. Two-colour staining analysis of freshly isolated human PBMC was performed using FITC-conjugated anti5H9, RD1-conjugated anti-CD3. Numbers indicate the relative percentages of positive cells within a quadrant. The result is representative of five separate experiments. (b) Reactivity of anti-CD9 MoAbs on H9 cells transfected with isolated cDNA of the 5H9 antigen. H9 parent cells as a negative control (a–d) or H9–5H9-1 cells transfected with isolated cDNA of 5H9 antigen (e–h) were stained with mouse IgG (a and e), anti5H9 (b and f), ALB6 (anti-CD9) (c and g), 4B4 (anti-CD29) (d and h) and FITC-conjugated goat antimouse IgG. (c) Western blotting of 5H9 antigen. H9–5H9-1 cells were lysed in lysis buffer. The lysates were separated on 12% SDS-PAGE under reducing (R) or non-reducing (NR) condition, and then immunoblotting was carried out with anti5H9 (blot). The positions of molecular weight markers are indicated on the left (MW). (d) Co-stimulatory effect of anti5H9 with the immobilized submitogenic dose of anti-CD3 on human peripheral blood T cells. Left: dose–response curve; right: time–course curve. Purified T cells at $2{\cdot}0 \times 10^5$ cells/well were stimulated with immobilized suboptimal concentration of anti-CD3 (OKT3) and the indicated concentrations of anti5H9 or anti-CD28 (4B10). Proliferation was assessed by [^{3}H]-thymidine (1 μCi/well) incorporation assay in the last 13 h of cultures. The result is representative of three independent experiments and expressed as mean cpm ± s.d. of triplicate cultures.

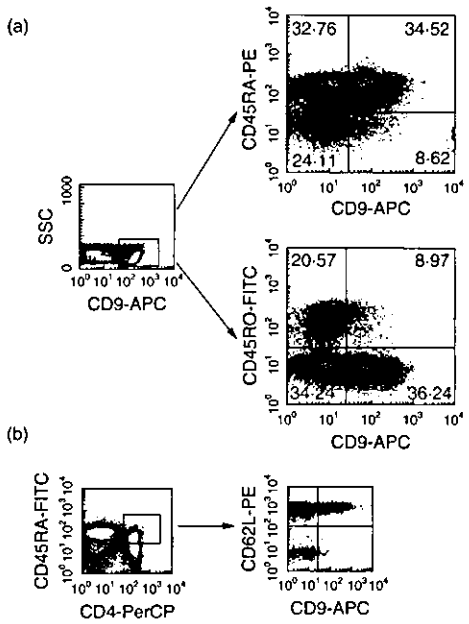

Fig. 2. Preferential expression of CD9 on CD4⁺CD45RA⁺ human naive T cells. CD9 defined by anti5H9 is present on a subpopulation of human peripheral blood T cells. Four-colour staining analysis of freshly isolated human PBMC was performed using FITC-conjugated anti-CD45RO, PE-conjugated anti-CD45RA, PerCP-conjugated anti-CD4, biotinylated anti5H9 and APC-conjugated streptavidin (a) and FITC-conjugated anti-CD45RA, PerCP-conjugated anti-CD4, PE-conjugated anti-CD62L, biotinylated anti5H9 and APC-conjugated streptavidin (b). Numbers indicate the relative percentages of positive cells within a quadrant. The result is representative of five separate experiments.

peripheral blood of healthy donors with HLA-DR53 by negative selection (CD45RA⁺ enriched T cells >95% positive, CD45RO⁺ enriched T cells >95% positive, Fig. 3a). As shown in Fig. 3b, we demonstrated a difference in the relative responsiveness of these two T cell subsets to a model of autoantigen or a recall antigen, as detected by T cell proliferation assay. CD45RA⁺ and CD45RO⁺ T cells responded preferentially to the recombinant beta$_2$-GPI and the recall antigen TT, respectively (Fig. 3b). In contrast, no significant difference was observed in the proliferative response to the non-specific stimulation of phytohaemagglutinin (PHA). After immunostaining with FITC-conjugated anti5H9, CD45RA⁺ T cells were sorted into CD9⁺ and CD9⁻ cells by flow cytometry, and then the proliferative response of CD9⁺CD45RA⁺ T cells and CD9⁻CD45RA⁺ T cells to beta$_2$-GPI was determined by T cell proliferation assay. As shown in Fig. 3c, CD9⁺CD45RA⁺ T cells incorporated significantly more [^{3}H]-thymidine than CD9⁻CD45RA⁺ T cells in response to beta$_2$-GPI (28977 ± 3859 cpm *versus* 15816 ± 409, $P < 0{\cdot}001$). In contrast, no difference was observed in the responsiveness of each population to PHA (38820 ± 5880 cpm *versus* 42576 ± 4615 $P = 0{\cdot}8$). Additionally, the responses of all CD45RA⁺ populations to TT were lower than that of unfractionated T cells. Therefore we could not assess the response of CD9⁺ or CD9⁻ populations in CD45RA⁺ T cells to the recall antigen TT. These results suggest that the CD45RA⁺ naive T cell population that expresses CD9 is the one that responds preferentially to beta$_2$-GPI.

Effect of anti5H9 on T cell proliferation induced by beta$_2$-GPI

Finally, to assess the potential involvement of CD9 molecule in the preferential responsiveness of CD4⁺CD45RA⁺ T cells to beta$_2$-GPI, the effect of anti5H9 on the proliferation of T cells to beta$_2$-GPI was investigated. As shown in Fig. 4a, anti5H9 significantly inhibited both beta$_2$-GPI- and TT-specific T cell proliferation in a dose-dependent manner, and anti-HLA DR (anti-L243) completely inhibited both responses. It has been reported that MoAbs against co-stimulatory molecule CD5, CD44 and CD11a, apart from CD28, all induce apoptosis of once-activated naive T cells [37]. Because it has also been reported that another tetraspanin molecule CD82 as well as CD9 induces apoptosis [42], we examined the effects of various MoAbs on beta$_2$-GPI induced T cell proliferation. In addition to anti5H9 (anti-CD9), anti-CD11a and anti-CD82 significantly inhibited the beta$_2$-GPI-induced T cell proliferation, while anti-CD28 and anti-CD29 did not (Fig. 4c). These results suggest that CD9 itself appears to play a key role in the specific proliferative response of naive and memory T cells to beta$_2$-GPI and TT, respectively.

DISCUSSION

In this study, we demonstrated that the newly developed anti5H9 MoAb targets human CD9, which is expressed preferentially on CD4⁺CD45RA⁺ naive T cells. Furthermore, this T cell population responds preferentially to recombinant beta$_2$-GPI, with anti5H9 MoAb being able to inhibit this beta$_2$-GPI-induced T cell proliferation and also TT-induced T cell proliferation, suggesting that the tetraspanin CD9 plays a important role both in the self-antigen- and recall antigen-induced T cell activation.

CD9 was described originally as a cell surface glycoprotein expressed on human pre-B cells and platelets but not on T cells [43,44]. While our preliminary experiments have demonstrated that several other anti-CD9 MoAbs hardly reacted with human peripheral blood T cells as detected by flow cytometric analysis (e.g. anti-ALB6 9·9%, anti-CLB 2·5%, anti72B6 0·9%), anti5H9 displayed a higher level of reactivity (anti5H9 26·6%). Moreover, others reported that murine CD9 defined by anti-KMC8·8 is only slightly expressed on murine T cells and B cells [45]. In contrast, it has been reported recently that murine CD9 recognized by anti9D3 is expressed on the cell surface of almost all T cells and B cells derived from murine thymus, spleen and lymph node [32,33,36]. Potential explanations for this observed discrepancy may involve differences in the relative affinity of anti-CD9 MoAbs or the specific epitope recognized by the various MoAbs. With our unique anti-CD9 MoAb, termed anti5H9, we have demonstrated clearly that CD9 is also expressed on human T cells, being restricted predominantly to the CD4⁺CD45RA⁺ naive T cell subset.

Concerning the role of tetraspanins in lymphocyte function, it has been shown that cross-linking of CD81 or CD82 on lymphocytes delivers co-stimulatory signals leading to cytokine production and modulation of cellular proliferation [15,24,46,47]. It has been also reported that murine CD9 defined by anti9D3 is a co-stimulatory molecule and is involved in activation-induced cell death [32,33,36]. Our present data show that CD9 also triggers a co-stimulatory signal in human T cells. However, the intensity of anti5H9-mediated T cell co-stimulation is relatively weaker than that induced by anti-CD28 in human T cells, potentially explained by the fact that CD9 expression is restricted to a selected T cell subset, while CD28 is present on almost all T cells. Moreover, we

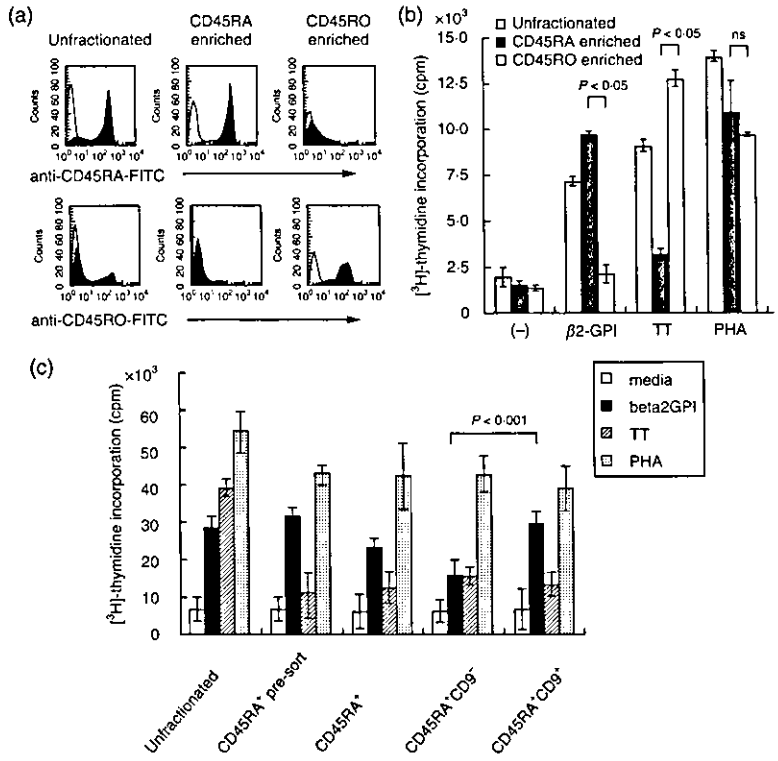

Fig. 3. Differential response of CD45RA$^+$ and CD45RO$^+$ T cells to recombinant beta$_2$-GPI and tetanus toxoid (TT). (a) CD45RA$^+$ T cells and CD45RO$^+$ T cells were enriched by negative selection, and their purity was analysed by flow cytometry; unfractionated: pre-enrichment, CD45RA enriched: CD45RA$^+$ T cells were enriched (>95% positive), CD45RO enriched: CD45RO$^+$ T cells were enriched (>95% positive). (b) The proliferative response to beta$_2$-GPI or TT was assessed with incorporation of [^{3}H]-thymidine. Media alone (–), beta$_2$-GPI (5 μg/ml), TT (5 ng/ml) and PHA (5 μg/ml) were used. The data are expressed as mean cpm ± s.d. of triplicate samples. Representative data of three separate experiments are shown. (c) Comparison between proliferative response of CD9$^+$CD45RA$^+$ T cells and CD9$^-$CD45RA$^+$ T cells to beta$_2$-GPI (5 μg/ml), TT (5 ng/ml) and PHA (5 μg/ml) by T cell proliferation assay; unfractionated (unfractionated T cells population), CD45RA$^+$ pre-sort (a population without anti5H9 treatment for cell sorting), and CD45RA$^+$ (a population with anti5H9 treatment for cell sorting) T cells were also examined. The data are expressed as mean cpm ± s.d. of sextuple samples. Representative data of three separate experiments are shown.

could not demonstrate clearly activation-induced cell death following CD9-mediated co-stimulation, in contrast to murine CD9 (data not shown). Thus, although there may be differences between human and murine T cells regarding CD9-associated apoptosis, our data would suggest that CD9 has a role in both human and murine T cell activation. While additional studies are required for the identification of a putative ligand for CD9 involved in T cell co-stimulation, it has already been reported that pregnant-specific glycoprotein 17 is a natural ligand for CD9 [48].

Our studies have focused on the functional property of CD9$^+$ T cells within the CD4$^+$CD45RA$^+$ T cell subset. It is reported that the CD4$^+$CD45RA$^+$ naive T cell subset proliferates maximally in the AMLR [2] and responds to self-antigens, such as heat shock protein 60 [11] or myelin basic protein [12]. Several attempts to identify autoreactive T cells have been reported previously [11,12,41,49]. Generally, most autoreactive T cells are deleted in the thymus by negative selection. However, some cells manage to evade the thymic selection process and migrate into the peripheral tissues in both patients with autoimmune diseases and healthy individuals. Hattori *et al.* have shown that CD4$^+$ T cells restricted by the HLA-DR53 alleles respond to reduced or recombinant beta$_2$-GPI but not native beta$_2$-GPI in both healthy individuals and patients with APS by presentation of its cryptic peptides [41]. Our present work using the same recombinant beta$_2$-GPI also showed that CD45RA$^+$-enriched T cells, but not CD45RO$^+$-enriched T cells, proliferated preferentially in response to recombinant beta$_2$-GPI, hence confirming that CD4$^+$CD45RA$^+$ T cells contain beta$_2$-GPI-reactive T cells. We demonstrated further that CD9$^+$CD4$^+$CD45RA$^+$ T cells represent the T cell population that responds maximally to recombinant beta$_2$-GPI. CD9 expression thus appears to be preferential to naive T cells with relatively high responsiveness to self-antigens. Meanwhile, the self-reactivity that causes autoimmune diseases is believed to be an integral physiological aspect of immunity based on the hypothesis that alterations in T cell-activation thresholds by self-ligands facilitate positive selection and regulate the level of self-reactivity in the periphery [50]. It is currently unclear whether CD9$^+$CD45RA$^+$ T cells play a role in pathogenic or physiological conditions. Regardless, our findings that anti-CD9 inhibited beta$_2$-GPI-mediated T cell response suggest that the CD9 molecule itself appears to be involved in the regulation of autoreactive T cells. As shown in Fig. 4c, anti-CD11a and anti-CD82 as well as anti-CD9 had an inhibitory effect on the beta$_2$-GPI-induced T cell proliferation, while

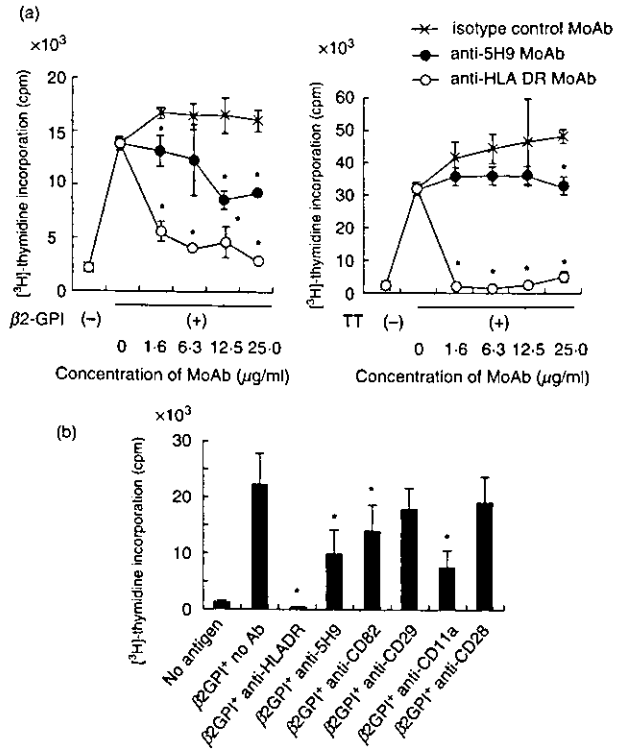

Fig. 4. Effect of anti5H9 on T cell proliferation induced by recombinant beta$_2$-GPI and TT. (a) The effect on proliferative response to recombinant beta$_2$-GPI (5 μg/ml) (left panel) and TT (5 ng/ml) (right panel) was assessed with various concentrations of MoAb, isotype-matched control MoAb (anti4B4), anti5H9 and anti-HLA DR MoAb (anti-L243). Data are expressed as mean cpm ± s.d. of triplicate samples. Representative data of three independent experiments are shown. *Significant inhibition compared to the culture with isotype-matched control MoAb ($P < 0.05$). (b) Inhibitory effect of anti5H9 (15 μg/ml) on beta$_2$-GPI-induced T cell proliferation was compared with those of various MoAbs (15 μg/ml). Data are expressed as mean cpm ± s.d. of sextuple samples. Representative data of three independent experiments are shown. *Significant inhibition compared to the culture without MoAb ($P < 0.05$).

anti-CD28 and anti-CD29 did not exhibit a similar effect. Although murine anti-CD9, anti-CD82 and anti-CD11a have been reported to trigger activation-induced cell death after co-stimulation [37,42], we could not detect it clearly after co-stimulation induced by anti5H9 (data not shown). Therefore, it is possible that anti-CD9 blocks beta$_2$-GPI-induced T cell activation by interfering with the cognitive interaction between antigen-presenting cells and T cells. Future work will determine the precise mechanisms involved in this process. This is the first study that demonstrates the functional significance of the tetraspanin CD9 expressed on human T cells. Further determination of the mechanism of co-stimulation through CD9, and identification of the CD9-ligand for co-stimulation, may lead to a detailed understanding of the regulation of autoreactive T cells and may provide insights into the development of immunotherapy for autoimmune diseases.

In summary, our data indicate that the tetraspanin CD9 was preferentially expressed on the population of CD4⁺CD45RA⁺ T cells. Meanwhile, the anti-CD9 antibody 5H9 provided a co-stimulatory signal to human peripheral T cells and inhibited both the recombinant beta$_2$-GPI- and the recall antigen TT-specific T cell proliferation, indicating that the CD9 molecule plays an important role in T cell activation. Elucidating the putative ligand for the CD9 molecule involved in T cell co-stimulation as well as the molecular mechanisms by which anti5H9 inhibits T cell activation will be necessary for a complete understanding of this interesting molecule.

ACKNOWLEDGEMENTS

This work was supported in part by National Institutes of Health grants AR33713, and by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology and from the Ministry of Health, Labor and Welfare of Japan. N. H. Dang is supported in part by a grant from the M. D. Anderson Cancer Center Physician–Scientist Program and the Gillson Longenbaugh Foundation. We thank Dr R. Mukasa, Dr T. Ohtsuki and Ms A. Kuribara for helpful discussion and technical support.

REFERENCES

1 Morimoto C, Letvin NL, Boyd AW *et al.* The isolation and characterization of the human helper inducer T cell subset. J Immunol 1985; **134**:3762–9.

2 Morimoto C, Letvin NL, Distaso JA, Aldrich WR, Schlossman SF. The isolation and characterization of the human suppressor inducer T cell subset. J Immunol 1985; **134**:1508–15.

3 Smith SH, Brown MH, Rowe D, Callard RE, Beverley PC. Functional subsets of human helper-inducer cells defined by a new monoclonal antibody, UCHL1. Immunology 1986; **58**:63–70.

4 Rudd CE, Morimoto C, Wong LL, Schlossman SF. The subdivision of the T4 (CD4) subset on the basis of the differential expression of L-C/T200 antigens. J Exp Med 1987; **166**:1758–73.

5 Takeuchi T, Schlossman SF, Morimoto C. The T4 molecule differentially regulating the activation of subpopulations of T4⁺ cells. J Immunol 1987; **139**:665–71.

6 Morimoto C, Matsuyama T, Rudd CE, Forsgren A, Letvin NL, Schlossman SF. Role of the 2H4 molecule in the activation of suppressor inducer function. Eur J Immunol 1988; **18**:731–7.

7 Sanders ME, Makgoba MW, Shaw S. Human naive and memory T cells: reinterpretation of helper-inducer and suppressor-inducer subsets. Immunol Today 1988; **9**:195–9.

8 Horgan KJ, Tanaka Y, Shaw S. Postthymic differentiation of CD4 T lymphocytes: naive versus memory subsets and further specialization among memory cells. Chem Immunol 1992; **54**:72–102.

9 Smolen JS, Chused TM, Novotny EA, Steinberg AD. The human autologous mixed lymphocyte reaction. III. Immune circuits. J Immunol 1982; **129**:1050–3.

10 Scheinecker C, Machold KP, Majdic O, Hocker P, Knapp W, Smolen JS. Initiation of the autologous mixed lymphocyte reaction requires the expression of costimulatory molecules B7-1 and B7-2 on human peripheral blood dendritic cells. J Immunol 1998; **161**:3966–73.

11 Ramage JM, Young JL, Goodall JC, Gaston JS. T cell responses to heat-shock protein 60: differential responses by CD4⁺ T cell subsets according to their expression of CD45 isotypes. J Immunol 1999; **162**:704–10.

12 Muraro PA, Pette M, Bielekova B, McFarland HF, Martin R. Human autoreactive CD4⁺ T cells from naive CD45RA⁺ and memory CD45RO⁺ subsets differ with respect to epitope specificity and functional antigen avidity. J Immunol 2000; **164**:5474–81.

13 Sanders ME, Makgoba MW, Sharrow SO *et al.* Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. J Immunol 1988; **140**:1401–7.

14 Morimoto C, Torimoto Y, Levinson G *et al.* 1F7, a novel cell surface

molecule, involved in helper function of CD4 cells. J Immunol 1989; **143**:3430–9.
15 Nojima Y, Hirose T, Tachibana K *et al.* The 4F9 antigen is a member of the tetra spans transmembrane protein family and functions as an accessory molecule in T cell activation and adhesion. Cell Immunol 1993; **152**:249–60.
16 Mukasa R, Homma T, Ohtsuki T *et al.* Core 2-containing O-glycans on CD43 are preferentially expressed in the memory subset of human CD4 T cells. Int Immunol 1999; **11**:259–68.
17 Sugita K, Tanaka T, Doshen JM, Schlossman SF, Morimoto C. Direct demonstration of the CD27 molecule involved in the negative regulatory effect on T cell activation. Cell Immunol 1993; **152**:279–85.
18 Torimoto Y, Rothstein DM, Dang NH, Schlossman SF, Morimoto C. CD31, a novel cell surface marker for CD4 cells of suppressor lineage, unaltered by state of activation. J Immunol 1992; **148**:388–96.
19 Mackay CR, Marston WL, Dudler L, Spertini O, Tedder TF, Hein WR. Tissue-specific migration pathways by phenotypically distinct subpopulations of memory T cells. Eur J Immunol 1992; **22**:887–95.
20 Wright MD, Tomlinson MG. The ins and outs of the transmembrane 4 superfamily. Immunol Today 1994; **15**:588–94.
21 Hemler ME, Mannion BA, Berditchevski F. Association of TM4SF proteins with integrins: relevance to cancer. Biochim Biophys Acta 1996; **1287**:67–71.
22 Maecker HT, Todd SC, Levy S. The tetraspanin superfamily: molecular facilitators. FASEB J 1997; **11**:428–42.
23 Shaw AR, Domanska A, Mak A *et al.* Ectopic expression of human and feline CD9 in a human B cell line confers beta 1 integrin-dependent motility on fibronectin and laminin substrates and enhanced tyrosine phosphorylation. J Biol Chem 1995; **270**:24092–9.
24 Lagaudriere-Gesbert C, Le Naour F, Lebel-Binay S *et al.* Functional analysis of four tetraspans, CD9, CD53, CD81, and CD82, suggests a common role in costimulation, cell adhesion, and migration: only CD9 upregulates HB-EGF activity. Cell Immunol 1997; **182**:105–12.
25 Miyake M, Koyama M, Seno M, Ikeyama S. Identification of the motility-related protein (MRP-1), recognized by monoclonal antibody M31–15, which inhibits cell motility. J Exp Med 1991; **174**:1347–54.
26 Miyake M, Nakano K, Ieki Y *et al.* Motility related protein 1 (MRP-1/CD9) expression: inverse correlation with metastases in breast cancer. Cancer Res 1995; **55**:4127–31.
27 Miyake M, Nakano K, Itoi SI, Koh T, Taki T. Motility-related protein-1 (MRP-1/CD9) reduction as a factor of poor prognosis in breast cancer. Cancer Res 1996; **56**:1244–9.
28 Mori M, Mimori K, Shiraishi T *et al.* Motility related protein 1 (MRP1/CD9) expression in colon cancer. Clin Cancer Res 1998; **4**:1507–10.
29 Le Naour F, Rubinstein E, Jasmin C, Prenant M, Boucheix C. Severely reduced female fertility in CD9-deficient mice. Science 2000; **287**:319–21.
30 Miyado K, Yamada G, Yamada S *et al.* Requirement of CD9 on the egg plasma membrane for fertilization. Science 2000; **287**:321–4.
31 Kaji K, Oda S, Shikano T *et al.* The gamete fusion process is defective in eggs of CD9-deficient mice. Nat Genet 2000; **24**:279–82.
32 Tai XG, Yashiro Y, Abe R *et al.* A role for CD9 molecules in T cell activation. J Exp Med 1996; **184**:753–8.
33 Tai XG, Toyooka K, Yashiro Y *et al.* CD9-mediated costimulation of TCR-triggered naive T cells leads to activation followed by apoptosis. J Immunol 1997; **159**:3799–807.
34 Miyazaki T, Muller U, Campbell KS. Normal development but differentially altered proliferative responses of lymphocytes in mice lacking CD81. EMBO J 1997; **16**:4217–25.
35 Knobeloch KP, Wright MD, Ochsenbein AF *et al.* Targeted inactivation of the tetraspanin CD37 impairs T-cell-dependent B-cell response under suboptimal costimulatory conditions. Mol Cell Biol 2000; **20**:5363–9.
36 Park CS, Yashiro Y, Tai XG *et al.* Differential involvement of a Fas-CPP32-like protease pathway in apoptosis of TCR/CD9-costimulated, naive T cells and TCR-restimulated, activated T cells. J Immunol 1998; **160**:5790–6.
37 Yashiro Y, Tai XG, Toyo-oka K *et al.* A fundamental difference in the capacity to induce proliferation of naive T cells between CD28 and other co-stimulatory molecules. Eur J Immunol 1998; **28**:926–35.
38 Kitamura T, Onishi M, Kinoshita S, Shibuya A, Miyajima A, Nolan GP. Efficient screening of retroviral cDNA expression libraries. Proc Natl Acad Sci USA 1995; **92**:9146–50.
39 Onishi M, Kinoshita S, Morikawa Y *et al.* Applications of retrovirus-mediated expression cloning. Exp Hematol 1996; **24**:324–9.
40 Deng HK, Unutmaz D, Kewa I, Ramani VN, Littman DR. Expression cloning of new receptors used by simian and human immunodeficiency viruses. Nature 1997; **388**:296–300.
41 Hattori N, Kuwana M, Kaburaki J, Mimori T, Ikeda Y, Kawakami Y. T cells that are autoreactive to beta2-glycoprotein I in patients with antiphospholipid syndrome and healthy individuals. Arthritis Rheum 2000; **43**:65–75.
42 Ono M, Handa K, Withers DA, Hakomori S. Motility inhibition and apoptosis are induced by metastasis-suppressing gene product CD82 and its analogue CD9, with concurrent glycosylation. Cancer Res 1999; **59**:2335–9.
43 Kersey JH, LeBien TW, Abramson CS, Newman R, Sutherland R, Greaves M. P-24: a human leukemia-associated and lymphohemopoietic progenitor cell surface structure identified with monoclonal antibody. J Exp Med 1981; **153**:726–31.
44 Dowell BL, Tuck FL, Borowitz MJ, LeBien TW, Metzgar RS. Phylogenetic distribution of a 24,000 dalton human leukemia-associated antigen on platelets and kidney cells. Dev Comp Immunol 1984; **8**:187–95.
45 Oritani K, Wu X, Medina K *et al.* Antibody ligation of CD9 modifies production of myeloid cells in long- term cultures. Blood 1996; **87**:2252–61.
46 Lebel-Binay S, Lagaudriere C, Fradelizi D, Conjeaud H. CD82, member of the tetra-span-transmembrane protein family, is a costimulatory protein for T cell activation. J Immunol 1995; **155**:101–10.
47 Shibagaki N, Hanada K, Yamaguchi S, Yamashita H, Shimada S, Hamada H. Functional analysis of CD82 in the early phase of T cell activation: roles in cell adhesion and signal transduction. Eur J Immunol 1998; **28**:1125–33.
48 Waterhouse R, Ha C, Dveksler GS. Murine CD9 is the receptor for pregnancy-specific glycoprotein 17. J Exp Med 2002; **195**:277–82.
49 Schmidt D, Goronzy JJ, Weyand CM. $CD4^+$ $CD7^-$ $CD28^-$ T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. J Clin Invest 1996; **97**:2027–37.
50 Grossman Z, Paul WE. Autoreactivity, dynamic tuning and selectivity. Curr Opin Immunol 2001; **13**:687–98.

Defective vasculogenesis in systemic sclerosis

Masataka Kuwana, Yuka Okazaki, Hidekata Yasuoka, Yutaka Kawakami, Yasuo Ikeda

Lancet 2004; 364: 603–10

See Comment page 561

Institute for Advanced Medical Research (M Kuwana MD, Y Okazaki, Y Kawakami MD) and **Department of Internal Medicine** (H Yasuoka MD, Y Ikeda MD), **Keio University School of Medicine, Tokyo, Japan**

Correspondence to:
Dr Masataka Kuwana, Institute for Advanced Medical Research, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
kuwanam@sc.itc.keio.ac.jp

Summary

Background Typical vascular features of systemic sclerosis include low capillary density and vascular obliteration. The formation and repair of blood vessels in adults involve vasculogenesis, which is mediated through the recruitment of bone-marrow-derived circulating endothelial precursors (CEP). We investigated whether vasculogenesis is impaired in patients with systemic sclerosis.

Methods Peripheral blood was obtained from 11 patients with systemic sclerosis, 11 with rheumatoid arthritis, and 11 healthy controls. Factors potentially affecting the CEP number were matched among the three groups. CEP (identified as circulating cells positive for CD34, CD133, and the type 2 receptor for vascular endothelial growth factor) were quantified by cell sorting and three-colour flow cytometry. The circulating concentrations of angiogenic factors were measured by ELISA. The potential of CEP to differentiate into endothelial cells was assessed by the upregulation of von Willebrand factor after in-vitro maturation treatment.

Findings The absolute number of CEP was much lower in patients with systemic sclerosis than in patients with rheumatoid arthritis or healthy controls (median 274 [IQR 178–395] *vs* 1154 [653–1524] and 1074 [713–1186] per 20 mL peripheral blood, respectively; $p<0·0001$ by Kruskal-Wallis test. Paradoxically, circulating concentrations of most angiogenic factors were significantly higher in patients with systemic sclerosis than in healthy controls. The proportion of CEP that differentiated into endothelial cells was significantly lower in patients with systemic sclerosis than in healthy controls ($p<0·0001$, Mann-Whitney test).

Interpretation Insufficient vascular repair machinery due to defective vasculogenesis might contribute to vasculopathy in systemic sclerosis.

Relevance to practice As well as providing an important insight into the pathogenesis of this disorder, these findings suggest that dysregulated vasculogenesis might be important in other disorders with abnormalities in vascular formation, including those with excessive formation of new vessels such as cancer and those with deficient vessel formation such as atherosclerosis. Circulating endothelial precursors could be a novel target for therapeutic strategies for ischaemic complications in patients with systemic sclerosis.

Introduction

Systemic sclerosis (or scleroderma) is a multiorgan disease characterised by excessive fibrosis and microvascular abnormalities.[1] The vasculopathy in the disorder mainly affects small arteries and capillaries and causes reduced blood flow and tissue ischaemia, which lead to clinical manifestations, such as Raynaud's syndrome, fingertip ulcers, and gangrene.[1] Morphological changes in the vessels of patients with this disease include lower than normal capillary density and obliteration of vessels due to intimal proliferation and fibrosis.[1] The vascular involvement in patients with systemic sclerosis is thought to be primarily induced by increased vascular injury occurring as a result of inflammatory immune processes, ischaemia-reperfusion reactions, and an imbalance between coagulation and fibrinolysis,[1] but the detailed sequence of the pathogenetic events remains unclear.

Recent studies have provided increasing evidence that the formation of new blood vessels in postnatal life does not result solely from the sprouting of pre-existing vessels (angiogenesis) but also involves the recruitment of bone-marrow-derived progenitors for endothelial cells (vasculogenesis).[2,3] These **circulating endothelial precursors (CEP)** have properties similar to those of embryonic angioblasts.[4] CEP can be identified by a characteristic surface phenotype that is positive for **CD34**, **CD133**, and **VEGFR-2**.[5] Postnatal vasculogenesis mainly contributes to vascular healing in response to vascular injury or ischaemia through the processes of rapid endothelialisation of denuded vessels and collateral vessel formation.[2,3,6] In this process, CEP home to the site of injury and work in concert with existing mature endothelial cells.[2,7]

In patients with systemic sclerosis, despite the reduced blood flow and tissue ischaemia, the formation of blood vessels seems to be insufficient to replace the damaged vessels.[1] The following findings support this idea: avascularity is the most prominent feature of nailfold capillaries in active disease;[8] $\alpha_v\beta_3$-positive newly formed blood vessels are almost completely absent from skin;[9] and angiography of many patients with late-stage systemic sclerosis indicates a lack of digital vessels. Therefore, we hypothesised that vasculopathy related to systemic sclerosis might be caused by defective vasculogenesis. To test this hypothesis, we have developed assay systems to assess the absolute numbers of CEP and their maturation potential, and we have used

Circulating endothelial precursors (CEP)
Migratory cells with capacity to circulate, proliferate, and differentiate into mature endothelial cells, but without typical mature endothelial morphology or markers or the ability to form vascular lumen. These cells can be identified by a characteristic surface phenotype positive for CD34, CD133, and VEGFR-2.

CD34
A transmembrane glycoprotein mainly expressed on haemopoietic stem cells and endothelial cells. This molecule serves as a ligand for L-selectin in endothelial cells, but its role in haemopoietic stem cells remains to be identified.

CD133
A pentaspan transmembrane glycoprotein that is specifically expressed on primitive cells with haemopoietic and endothelial differentiation potential and is used for identification of early haemopoietic stem cells and endothelial progenitors. The specific function and potential ligands are currently unknown.

Vascular endothelial growth factor receptor type 2 (VEGFR-2)
A receptor tyrosine kinase also known as Flk-1 and KDR that is a high-affinity receptor for vascular endothelial growth factor and has a crucial role in vasculogenesis and angiogenesis. The expression is restricted to vascular endothelial cells and their progenitors.

	Systemic sclerosis (n=11)	Rheumatoid arthritis (n=11)	Healthy controls (n=11)
Mean (SD) age at examination, years	57·7 (11·8)	59·1 (12·0)	52·7 (10·6)
Median (IQR) disease duration, years	10·0 (7·0–11·0)	9·0 (1·5–14·0)	..
Postmenopausal	7 (64%)	7 (64%)	7 (64%)
Current smokers	1 (9%)	1 (9%)	1 (9%)
Hypertensive	1 (9%)	2 (18%)	1 (9%)
Hypercholesterolaemic	2 (18%)	3 (27%)	2 (18%)
Receiving corticosteroids	5 (45%)	5 (45%)	..

Data are number of participants unless otherwise stated.

Table 1: **Clinical characteristics of study participants**

these tests to examine the quantity and function of CEP in patients with systemic sclerosis.

Methods

Patients

We studied 11 female patients with systemic sclerosis, all of whom met the American College of Rheumatology preliminary criteria.[10] Patients with symptoms that overlapped those of other connective-tissue diseases were excluded. Controls matched for age and sex were 11 female patients with rheumatoid arthritis, as a systemic inflammatory disease control, and 11 healthy women. All patients with rheumatoid arthritis met the American College of Rheumatology classification criteria[11] and had evidence of active disease according to the published criteria.[12] We studied only women because the prevalence of systemic sclerosis is five to ten times higher in women than in men, and the number and function of CEP are potentially influenced by sex because physiological new blood formation occurs prominently in the endometrium. Patients were selected from those who visited our outpatient clinic during a 2-month period (November and December, 2002) and were willing to donate blood samples for this study. All peripheral-blood samples were obtained on several occasions between November, 2002, and February, 2003. This study was approved by the Keio University Institutional Review Boards, and written informed consent was obtained from all participants.

Procedures

For quantification of CEP, peripheral-blood mononuclear cells were isolated from 20 mL heparinised venous blood by Lymphoprep (Nycomed Pharma AS, Oslo, Norway) density-gradient centrifugation. CD34-positive cells were enriched from peripheral-blood mononuclear cells by an immunomagnetic technique with a monoclonal antibody to CD34 coupled to magnetic beads (CD34 MicroBeads; Miltenyi Biotech, Bergisch Gladbach, Germany). The CD34-cell-enriched fraction was then incubated with a monoclonal antibody to VEGFR-2 (KDR-1; Sigma, St Louis, MO, USA) and biotin-conjugated goat antibody to mouse IgG $F(ab')_2$ (Immunotech, Marseille, France), followed by additional staining with a fluorescein-isothiocyanate-conjugated monoclonal antibody to CD34 (AC136), phycoerythrin-conjugated monoclonal antibody to CD133 (AC133/2; Miltenyi Biotech), and streptavidin-PC5 (Immunotech). Control cells were also prepared by incubation with fluorescence-labelled isotype-matched monoclonal antibodies. The cells were then analysed by three-colour flow cytometry with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Viable cells were identified by gating on forward and side scatters, and the expression of CD133 and VEGFR-2 was assessed on gated CD34-positive cells. CEP were identified as cells positive for CD34, VEGFR-2, and CD133.[5] The total number of viable cells in the CD34-cell-enriched fraction was assessed from its ratio to the FlowCount microbeads (Beckman-Coulter, Hialeah, FL, USA), and we calculated the absolute numbers of cells in 20 mL peripheral blood that were positive for: CD34; both CD34 and CD133; or CD34, CD133, and VEGFR-2 (CEP); and the number positive for CD34 and VEGFR-2 but negative for CD133. A consistent detector sensitivity, compensation setting, and scatter gate set were used to analyse all samples. Because of variability in staining intensity across the samples, the cut-off setting was decided on in individual samples on the basis of the staining intensity of control cells incubated with a series of isotype-matched monoclonal antibodies. All procedures were done by the same operator without knowledge of the sample identity. The coefficients of variation for five separate assays of CEP in the samples from two healthy individuals were 14·2% and 10·7%.

CD133-positive cells were isolated from at least 5×10^7 peripheral-blood mononuclear cells by the MACS sorting system with magnetic beads coupled with monoclonal antibody to CD133 (Miltenyi Biotech). Flow-cytometric analysis showed that the cell fractions positive for CD133 consistently contained more than 85% such cells. The CD133-positive cells were resuspended in Dulbecco's modified Eagle's medium (Sigma) and cultured on fibronectin-coated chamber slides (Beckton-Dickinson) overnight. Human umbilical-vein endothelial cells (Clonetics, San Diego, CA, USA) were used as a mature endothelial-cell control. The adherent cells were then fixed with 4% paraformaldehyde, and incubated for 30 min with rabbit polyclonal antibodies to VEGFR-2 or Tie-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in combination with mouse monoclonal antibodies to CD31 (WM-59), VEGFR-2 (KDR-1; both Sigma), CD45 (PD7/26), von Willebrand factor (2F2-A9; both Dako, Carpinteria, CA, USA), CD146 (P1H12), or vascular endothelium cadherin (BV6; both Chemicon International, Temecula, CA, USA). The cells were subsequently incubated with rabbit-specific IgG conjugated to AlexaFluor 568 and mouse-specific IgG conjugated to AlexaFluor 488 (Molecular Probes, Eugene, OR, USA). To assess the uptake of acetylated LDL, cultured adherent cells were incubated with acetylated LDL labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethy-

lindocarbocyanine (Molecular Probes) for 1 h at 37°C, then with a rabbit antibody to VEGFR-2 and rabbit-specific IgG conjugated to AlexaFluor 488. The negative controls were cells incubated with normal mouse IgG and rabbit IgG instead of the primary antibodies. Nuclei were stained with TO-PRO-3 (Molecular Probes). The stained cells were examined with a confocal laser fluorescence microscope (LSM5 PASCAL; Carl-Zeiss, Göttingen, Germany). At least ten cells that stained for VEGFR-2 were investigated for expression of CD31, vascular-endothelium cadherin, CD146, von Willebrand factor, CD45, and Tie-2 and for uptake of acetylated LDL.

The concentrations of vascular endothelial growth factor in heparinised platelet-poor plasma and of basic fibroblast growth factor, hepatocyte growth factor, and erythropoietin in serum were measured by specific ELISA kits (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

To assess the potential of CEP to differentiate into mature endothelial cells in response to angiogenic stimuli, the cells were cultured as described previously[13–15] with several modifications. CD133-positive cells and CD133-negative cells were separated from more than 5×10^7 peripheral-blood mononuclear cells by the MACS sorting system. CD133-positive cells were then plated with CD133-negative cells at a ratio of 1 to 50 on fibronectin-coated chamber slides at an overall density of 5×10^1 cells/μL. The cells were cultured in endothelial cell basal medium 2 (Clonetics) supplemented with MV SingleQuots containing fetal bovine serum, vascular endothelial growth factor, basic fibroblast growth factor, epidermal growth factor, insulin-like growth factor 1, heparin, and ascorbic acid for 5 days. Because CEP are known to form late-outgrowth colonies after 9 days in vitro,[15] proliferation of CEP is negligible in this short-term culture. The cells were fixed and incubated with mouse antibody to VEGFR-2 (KDR-1) or monoclonal antibody to von Willebrand factor (2F2-A9) followed by incubation with AlexaFluor 568 mouse-specific IgG and then with fluorescein-isothiocynate-conjugated mouse monoclonal antibody to CD45 (PD7/26). The negative controls were cells incubated with normal mouse IgG instead of the primary antibody. The cells were examined with a confocal laser fluorescence microscope. The individual staining was carried out in two to four wells, and the mean number of cells that lacked CD45 expression but expressed VEGFR-2 or von Willebrand factor was counted. The proportion of mature CEP obtained in this in-vitro maturation treatment was calculated as the ratio of CD45-negative, von-Willebrand-factor-positive cells to CD45-negative, VEGFR-2-positive cells.

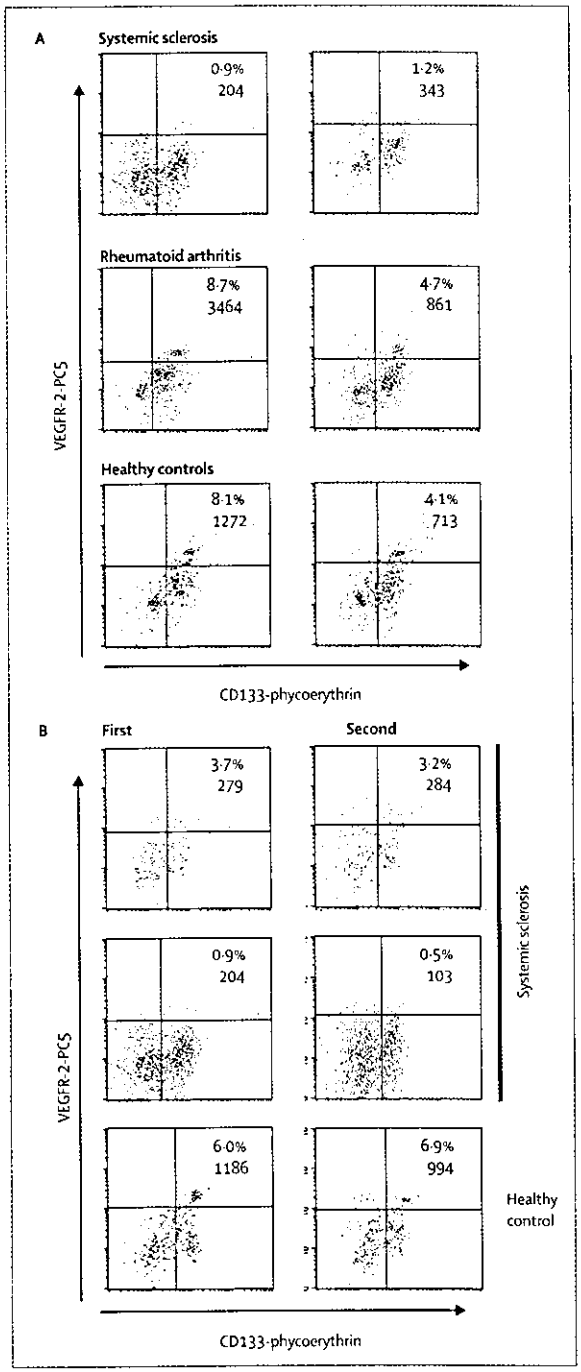

Figure 1: **Detection of CEP**
A: Detection of CEP by flow cytometry in two representative participants from each study group. The upper right section of individual dot-plot images indicates CD34-positive, CD133-positive, VEGFR-2-positive CEP. The percentage of CEP in the gated CD34-positive cells and the absolute number of CEP in 20 mL peripheral blood are shown in each panel. B: Serial measurement of CEP beyond a 3-month period in two patients with systemic sclerosis and one healthy control.

Statistical methods

No formal sample-size calculation was done before the study began. Since the number and function of CEP are potentially influenced by the weather, we thought that all samples should be obtained in one winter season. This constraint limited our sample size to 11 patients in

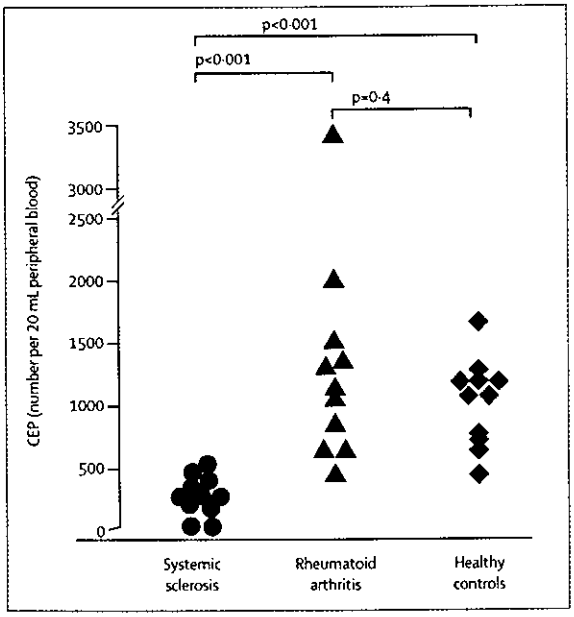

Figure 2: Absolute CEP numbers in 20 mL peripheral blood from each participant
p values by Kruskal-Wallis test followed by groupwise comparisons with Wilcoxon's rank sum test.

each group. Categorical variables were compared by use of the χ^2 test. The Shapiro-Wilk test was used to assess the normality of continuous data. Comparison between any two groups was by unpaired *t* test for normally distributed data or non-parametric Mann-Whitney test for non-normally distributed data. Multiple group comparison was done by one-way ANOVA for data with normal distribution. The Kruskal-Wallis test was used for data with non-normal distribution. When the p value for this overall comparison was significant (<0·05), post-hoc pairwise comparisons were done with the Dunnett's *t* test for data with normal distribution or Wilcoxon's rank sum test for data with non-normal distribution. The p values of the post-hoc pairwise comparisons were subsequently adjusted by Bonferroni correction. Correlation between two variables was tested by Pearson's test.

Role of the funding source

The sponsor of the study had no role in the study design; collection, analysis, or interpretation of data; writing of the report; or the decision to submit it for publication.

Results

The clinical characteristics of the participants are summarised in table 1. There was no difference in age at examination among the three groups. The disease duration was similar in patients with systemic sclerosis and those with rheumatoid arthritis, and both groups included patients with early and late disease. All the participants were female, and factors potentially affecting the number of CEP, including age, menstruation status, smoking, hypertension, and hypercholesterolaemia,[16] were matched among the three groups. No participant had diabetes mellitus or coronary-artery disease or was taking statins. No patient with systemic sclerosis or rheumatoid arthritis had received cytotoxic drugs or ciclosporin at any time during their illness, but five patients with systemic sclerosis and five with rheumatoid arthritis were receiving low-dose corticosteroids (<10 mg daily) at the time of blood sampling. Of the patients with systemic sclerosis, six had diffuse cutaneous involvement. The mean modified Rodnan's total skin score[17] at examination was 11·4 (SD 6·6). All patients had Raynaud's syndrome and seven had pitting scars. Two patients had active fingertip ulcers on several digits, and in one amputation of the right foot was necessary owing to progressive gangrene.

Flow-cytometric analyses for the staining of CD133 and VEGFR-2 on the gated CD34-positive cells in representative participants are shown in figure 1. CEP, identified as cells positive for both CD133 and VEGFR-2, were clearly visible in samples from two patients with rheumatoid arthritis and two healthy controls but were scarcely detected in samples from two patients with systemic sclerosis. The absolute numbers of CEP were much lower in patients with systemic sclerosis than in those with rheumatoid arthritis and healthy controls (figure 2) but similar in patients with rheumatoid

	Systemic sclerosis (n=11)	Rheumatoid arthritis (n=11)	Healthy controls (n=11)	p (for overall comparison)
Absolute number of cells in 20 mL peripheral blood				
Mean (SD) CD34-positive	12557 (9974)*	15 225 (10135)	25892 (9552)	0·009
Mean (SD) CD34-positive, CD133-positive	4668 (3691)†	8109 (5468)	13768 (7023)	0·002
Median (IQR) CD34-positive, CD133-positive, VEGFR-2-positive (CEP)	274 (178–395)‡§	1154 (653–1524)	1074 (713–1186)	<0·0001
Median (IQR) CD34-positive, CD133-negative, VEGFR-2-positive	79 (23–218)	76 (37–180)	53 (31–64)	0·3
Median (IQR) circulating concentrations of angiogenic factors				
Vascular endothelial growth factor, ng/L	34·5 (11·0–53·1)*¶	76·0 (59·4–112·5)†	10·0 (10·0–16·1)	<0·0001
Basic fibroblast growth factor, ng/L	33·0 (21·7–36·1)‡	24·0 (19·4–26·9)‡	9·0 (8·6–9·7)	<0·0001
Hepatocyte growth factor, ng/L	1330 (1062–1466)†	1337 (1064–1441)†	741 (691–900)	<0·0001
Erythropoietin, IU/L	9·7 (3·5–11·1)	13·0 (7·7–15·8)*	6·2 (5·2–8·2)	0·01

p for the overall group comparison was assessed by one-way ANOVA for data with normal distribution or the Kruskal-Wallis test for data with non-normal distribution. When post-hoc pairwise comparisons followed by the Bonferroni correction were made, patients with systemic sclerosis or rheumatoid arthritis differed significantly different from healthy controls at: *p<0·05; †p<0·01; or ‡p<0·001. Patients with systemic sclerosis differed significantly different from those with rheumatoid arthritis at: §p<0·001; or ¶p<0·05.

Table 2: Absolute numbers of CEP and concentrations of angiogenic factors in circulation of participants

arthritis and healthy controls (table 2). Repeated measurements for five patients with systemic sclerosis and three healthy controls found stable numbers of CEP beyond a 3-month period in all cases (figure 1). In the patients with systemic sclerosis, the CEP number was not associated with the disease subset (diffuse *vs* limited), disease duration, or total skin score, but it was significantly lower in the seven patients with pitting scars than in the four without this feature (204 [IQR 110–276] *vs* 403 [326–479], p=0·03 by Mann-Whitney test). In addition, active fingertip ulcers were observed exclusively in patients with the lowest and second lowest numbers of CEP, which were less than 5% of the mean in healthy controls. The absolute numbers of cells positive for the three markers of interest in the three groups are also listed in table 2. The numbers of circulating CD34-positive and CD34-positive, CD133-positive cells were significantly lower in patients with systemic sclerosis or rheumatoid arthritis than in healthy controls. This difference may reflect the impaired haemopoiesis that is reported in patients with rheumatoid arthritis.[18] The number of CD34-positive, CD133-negative, VEGFR-2-positive cells containing circulating mature endothelial cells[5] was slightly but not significantly higher in patients with systemic sclerosis or rheumatoid arthritis than in healthy controls.

The CEP phenotype was assessed by fluorescence double-immunostaining of adherent CD133-positive cells of three patients with systemic sclerosis and three healthy controls. Representative images obtained from a healthy control are shown in figure 3. More than 80% of the adherent CD133-positive, VEGFR-2-positive cells coexpressed CD31 and Tie-2 and were positive for uptake of acetylated LDL, but they lacked expression of the haemopoietic marker CD45. This feature was seen in the control human umbilical-vein endothelial cells and was consistent with previous reports.[5,6] By contrast, expression of vascular-endothelium cadherin and a mature endothelial-cell marker CD146 in CEP was faint and not distributed on the cell surface. Furthermore, CEP lacked expression of von Willebrand factor, another marker of mature endothelial cells. There was no difference in the phenotype of CEP between patients with systemic sclerosis and healthy controls.

Angiogenic factors are known to potentiate the proliferation and recruitment of CEP from the bone marrow.[19,20] To test whether the lower numbers of CEP in patients with systemic sclerosis were due to the impaired production of angiogenic factors, circulating concentrations of vascular endothelial growth factor, basic fibroblast growth factor, hepatocyte growth factor, and erythropoietin were measured in the three study groups (table 2). Concentrations of all these angiogenic factors were higher in patients with systemic sclerosis than in healthy controls, as reported previously,[21,22] and differences in vascular endothelial growth factor, basic fibroblast growth factor and hepatocyte growth factor were significant. The concentrations of basic fibroblast growth factor and hepatocyte growth factor were similar in patients with systemic sclerosis and in those with rheumatoid arthritis, but the concentration of vascular endothelial growth factor was significantly higher in patients with rheumatoid arthritis than in those with systemic sclerosis. When relations between circulating concentrations of individual angiogenic factors and the CEP number were examined, only the concentration of

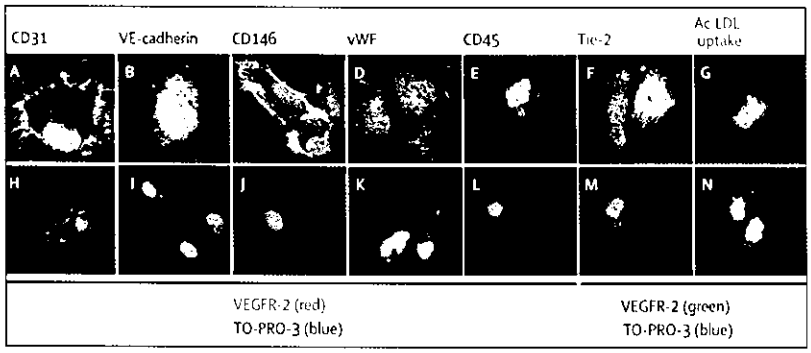

Figure 3: **Phenotypic characterisation of CEP**
Human umbilical-vein endothelial cells (A–G) and adherent CD133-positive cells from a healthy control (H–N) were fixed, stained with antibodies to VEGFR-2 (A–E and H–L; red, and F, G, M, and N; green) in combination with various antibodies, and examined with a confocal laser fluorescence microscope. VE=vascular endothelium; vWF=von Willebrand factor;.Ac-LDL=acetylated LDL. Nuclei were stained with TO-PRO-3. Original magnification, ×630.

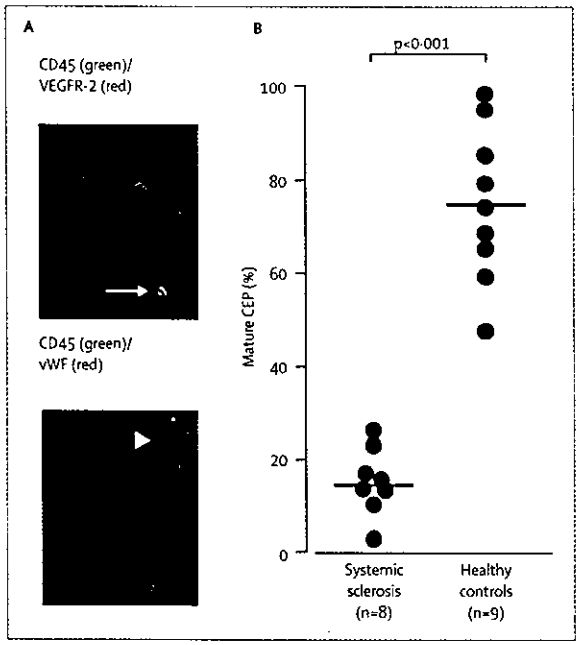

Figure 4: **In-vitro differentiation of CEP into mature endothelial cells in response to angiogenic stimuli**
A: CD45-negative, VEGFR-2-positive cells originated from CEP (arrow), and CD45-negative, von-Willebrand-factor-positive cells were mature endothelial cells that differentiated from CEP (arrowhead). CD45-positive cells lacking expression of VEGFR-2 or von Willebrand factor (vWF) were derived from circulating monocytes. Original magnification, ×100. B: Proportion of CEP with maturation potential in response to in-vitro angiogenic treatment. Comparisons were made by unpaired t test. Bar indicates mean value.

basic fibroblast growth factor was negatively correlated with the CEP number (r=–0·76, p=0·006).

In a preliminary experiment, we showed that CEP were induced to express von Willebrand factor by the in-vitro maturation treatment; therefore, the upregulated expression of this factor was used to assess the maturation potential of CEP as a marker for mature endothelial cells. Freshly isolated CD133-positive cells were cultured with a combination of angiogenic factors in the presence of CD133-negative cells, which were a required source of additional stimuli necessary for in-vitro maturation. We could not obtain sufficient CD133-positive cells for this assay from three patients with systemic sclerosis who had extremely low numbers of CEP. None of the adherent cells from three healthy donors expressed von Willebrand factor after overnight culture, indicating negligible contamination of mature endothelial cells in these cultures. After a 5-day culture, nearly all the adherent cells expressed CD45, and these were likely to have originated from monocytes in the CD133-negative cell fraction. By contrast, CD45-negative, VEGFR-2-positive cells and CD45-negative, von-Willebrand-factor-positive cells, which originated from CEP, represented less than 1% of the total adherent cells (figure 4). The proportion of CEP that differentiated in vitro into endothelial cells expressing von Willebrand factor was significantly lower in eight patients with systemic sclerosis than in nine healthy controls (mean 15·3% [SD 7·2] *vs* 74·5% [16·6], p<0·001 by unpaired t test).

Discussion

We found that patients with systemic sclerosis had much lower numbers of CEP than healthy controls. Patients with extremely low CEP numbers were likely to have profound vascular involvement, such as pitting scars and fingertip ulcers. An overall deficiency in CD34-positive cells and CD34-positive, CD133-positive cells in systemic sclerosis and rheumatoid arthritis suggests impairment of haemopoiesis in these connective-tissue diseases; however, a deficiency in CEP was detected in systemic sclerosis but not in rheumatoid arthritis. Although the CEP phenotype was similar for cells isolated from patients with systemic sclerosis and healthy controls, CEP in patients with systemic sclerosis were functionally impaired and resistant to the in-vitro maturation treatment. In view of the important role of CEP in postnatal vasculogenesis, our findings strongly suggest that the failure of new blood-vessel formation in response to vascular damage in patients with systemic sclerosis can be explained by defective vasculogenesis. Because of the lack of specific markers to distinguish CEP from mature endothelial cells and certain haemopoietic cells accurately, the cells expressing CD34, CD133, and VEGFR-2 analysed in our assays might include non-endothelial precursors or miss a subset of primitive cells with capacity to differentiate into endothelial cells.

The rapid endothelialisation of denuded injured vessels is essential to avoid fatal complications, such as bleeding and thrombosis. When vascular injury occurs, many growth factors are rapidly produced by the damaged vessels and adherent activated platelets to promote vascularisation.[6,19] After acute vascular insult, including burns and coronary-artery bypass grafting, there is a rapid rise in plasma concentrations of vascular endothelial growth factor followed by mobilisation of CEP during the first 6–12 h; and the concentration of vascular endothelial growth factor returns to the basal value within 48–72 h.[6] In a setting of defective vasculogenesis such as systemic sclerosis, this tissue response might be accelerated and prolonged, and sustained production of the growth factors would induce the proliferation of vascular smooth-muscle cells and fibroblasts in the intima and excessive deposition of extracellular matrix. This process might be increased by activation of inflammation and coagulation systems, which is secondarily induced by endothelial damage and dysfunction. This pathogenetic process might not be restricted to vessel walls but might also activate fibroblasts in the surrounding tissues to induce excessive fibrosis in the dermis and other organs. Patients with rheumatoid arthritis, who have raised concentrations of circulating angiogenic factors similar to those in patients with systemic sclerosis, show prominent formation of new blood vessels on the synovium, which is now thought to be a preferential therapeutic target.[23]

The mechanism that causes the deficiency of CEP in systemic sclerosis is unknown. CEP in peripheral blood could be destroyed or their production in the bone marrow impaired. Several in-vitro studies with serum antibodies from patients with systemic sclerosis have found that endothelial-cell apoptosis is induced by antibodies to endothelial cells through antibody-dependent cell-mediated cytotoxicity[24] or via interaction with a specific cell-surface molecule.[25] In addition, cytomegalovirus is thought to be involved in the pathogenesis of systemic sclerosis, since it can infect endothelial cells.[26] CEP and mature endothelial cells share many phenotypic and functional properties,[5] so CEP might be a preferential target of these cytotoxic activities.

However, the high concentrations of vascular endothelial growth factor, basic fibroblast growth factor, and hepatocyte growth factor we found in the circulation of patients with systemic sclerosis strongly suggest that CEP and their stem cells in the bone marrow did not respond adequately to these angiogenic factors. An imbalance between angiogenic and angiostatic factors is a possible cause, but the concentration of endostatin, an angiostatic factor generated from collagen type XVIII, is not increased in patients with systemic sclerosis compared with healthy controls.[21] However, the possibility that other angiostatic factors suppress

maturation of CEP or their stem cells cannot be excluded. Furthermore, continuous endothelial injury might lead to eventual depletion of CEP, as suggested in patients with multiple risk factors for atherosclerosis,[16] although two patients with early systemic sclerosis (disease duration <3 years) in our study had low CEP numbers (279 and 463 per 20 mL). Further studies of a large number of patients with early and late disease are needed to test this possibility. An alternative hypothesis is that CEP, their stem cells, or both, are functionally altered and are intrinsically hyporesponsive to angiogenic stimuli, since the CEP in patients with systemic sclerosis showed impaired maturation potential in vitro induced by a combination of angiogenic stimuli. The negative correlation between the circulating concentrations of basic fibroblast growth factor and the number of CEP in patients with systemic sclerosis supports this hypothesis. Similar CEP dysfunction has also been reported in diabetes mellitus, a disease known to cause vasculopathy similar to that of systemic sclerosis.[27] Further functional characterisation of the CEP in patients with systemic sclerosis is necessary for these possibilities to be examined, but the low frequency of CEP in the circulation, especially in these patients, makes these studies difficult.

Conventional treatment for vascular involvement in patients with systemic sclerosis is limited to non-specific vasodilator agents, and little success has been achieved by these therapies, which solely target vascular dysfunction.[28] The findings of this study suggest that strategies that mobilise CEP into the circulation might have therapeutic potential in the vasculopathy of systemic sclerosis. Granulocyte colony-stimulating factor is known to mobilise CEP from the bone marrow.[5] Moreover, although the concentrations of vascular endothelial growth factor and basic fibroblast growth factor are already high in patients with systemic sclerosis, a further increase obtained by the administration of these factors, either as recombinant proteins or by gene transfer,[29,30] could augment vasculogenesis as a therapeutic option for patients with severe ischaemic disease. Statins stimulate CEP kinetics and increase the number of CEP in circulation.[31] Therapeutic effects of these potential strategies will depend on the mechanism for the defective vasculogenesis in systemic sclerosis.

Thus, our findings lead us to propose a new theory that insufficient vascular repair machinery through defective vasculogenesis contributes to the vascular involvement in systemic sclerosis. Since the number of patients examined in this study was small, further studies are necessary to confirm our theory. Investigation of functional changes in postnatal vasculogenesis in patients with systemic sclerosis could be useful for clarifying the pathogenesis of this disease and developing novel therapeutic interventions for ischaemic complications in patients with systemic sclerosis.

Contributors

M Kuwana had the original idea for the study, designed the study, and participated in experimental procedures and data analysis. Y Okazaki did most of the experimental procedures. H Yasuoka and Y Kawakami participated in some of the experimental procedures and the data analysis. Y Ikeda recruited the patients and supervised the study. All authors contributed to the writing of the report.

Conflict of interest statement

None declared.

Acknowledgments

We thank M Kajihara for assistance in statistical analysis. This work was supported by a grant from the Japanese Ministry of Health, Labor and Welfare.

References

1 LeRoy EC. Systemic sclerosis: a vascular perspective. *Rheum Dis Clin North Am* 1996; 22: 675–94.
2 Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 1999; 85: 221–28.
3 Bhattacharya V, McSweeney PA, Shi Q, et al. Enhanced endothelialization and microvessel formation in polyester grafts seeded with CD34+ bone marrow cells. *Blood* 2000; 95: 581–85.
4 Caprioli A, Jaffredo T, Gautier R, Dubourg C, Dieterlen-Lievre F. Blood-borne seeding by hematopoietic and endothelial precursors from the allantois. *Proc Natl Acad Sci USA* 1998; 95: 1641–46.
5 Peichev M, Naiyer AJ, Pereira D, et al. Expression of VEGFR-2 and AC133 by circulating human CD34+ cells identifies a population of functional endothelial precursors. *Blood* 2000; 95: 952–58.
6 Gill M, Dias S, Hattori K, et al. Vascular trauma induces rapid but transient mobilization of VEGFR2+AC133+ endothelial precursor cells. *Circ Res* 2001; 88: 167–74.
7 Murayama T, Tepper OM, Silver M, et al. Determination of bone marrow-derived endothelial progenitor cell significance in angiogenic growth factor-induced neovascularization in vivo. *Exp Hematol* 2002; 30: 967–72.
8 Chen ZY, Silver RM, Ainsworth SK, Dobson RL, Rust P, Maricq HR. Association between fluorescent antinuclear antibodies, capillary patterns, and clinical features in scleroderma spectrum disorders. *Am J Med* 1984; 77: 812–22.
9 Mackiewicz Z, Sukura A, Povilenaite D, et al. Increased but imbalanced expression of VEGF and its receptors has no positive effect on angiogenesis in systemic sclerosis skin. *Clin Exp Rheumatol* 2002; 20: 641–46.
10 Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 1980; 23: 581–90.
11 Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315–24.
12 Maini R, St Clair EW, Breedveld F, et al. Infliximab (chimeric anti-tumour necrosis factor alpha monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. *Lancet* 1999; 354: 1932–39.
13 Gehling UM, Ergun S, Schumacher U, et al. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. *Blood* 2000; 95: 3106–12.
14 Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; 275: 964–67.
15 Lin Y, Weisdorf DJ, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest* 2000; 105: 71–77.
16 Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001; 89: E1–7.

17 Clements PJ, Medsger TA Jr. Organ involvement: skin. In: Clements PJ, Furst DE, eds. Systemic sclerosis. Baltimore: Williams and Wilkins, 1996: 389–407.

18 Papadaki HA, Kritikos HD, Gemetzi C, et al. Bone marrow progenitor cell reserve and function and stromal cell function are defective in rheumatoid arthritis: evidence for a tumor necrosis factor alpha-mediated effect. *Blood* 2002; **99**: 1610–19.

19 Takahashi T, Kalka C, Masuda H, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* 1999; **5**: 434–38.

20 Heeschen C, Aicher A, Lehmann R, et al. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood* 2003; **102**: 1340–46.

21 Distler O, Del Rosso A, Giacomelli R, et al. Angiogenic and angiostatic factors in systemic sclerosis: increased levels of vascular endothelial growth factor are a feature of the earliest disease stages and are associated with the absence of fingertip ulcers. *Arthritis Res* 2002; **4**: R11.

22 Kawaguchi Y, Harigai M, Fukasawa C, Hara M. Increased levels of hepatocyte growth factor in sera of patients with systemic sclerosis. *J Rheumatol* 1999; **26**: 1012–13.

23 Weber AJ, de Bandt M. Angiogenesis: general mechanisms and implications for rheumatoid arthritis. *Joint Bone Spine* 2000; **67**: 366–83.

24 Sgonc R, Gruschwitz MS, Boeck G, Sepp N, Gruber J, Wick G. Endothelial cell apoptosis in systemic sclerosis is induced by antibody-dependent cell-mediated cytotoxicity via CD95. *Arthritis Rheum* 2000; **43**: 2550–62.

25 Lunardi C, Bason C, Navone R, et al. Systemic sclerosis immunoglobulin G autoantibodies bind the human cytomegalovirus late protein UL94 and induce apoptosis in human endothelial cells. *Nat Med* 2000; **6**: 1183–86.

26 Pandey JP, LeRoy EC. Human cytomegalovirus and the vasculopathies of autoimmune diseases (especially scleroderma), allograft rejection, and coronary restenosis. *Arthritis Rheum* 1998; **41**: 10–15.

27 Tepper OM, Galiano RD, Capla JM, et al. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* 2002; **106**: 2781–86.

28 Schachna L, Wigley FM. Targeting mediators of vascular injury in scleroderma. *Curr Opin Rheumatol* 2002; **14**: 686–93.

29 Rajagopalan S, Mohler E III, Lederman RJ, et al. Regional angiogenesis with vascular endothelial growth factor (VEGF) in peripheral arterial disease: design of the RAVE trial. *Am Heart J* 2003; **145**: 1114–18.

30 Freedman SB, Isner JM. Therapeutic angiogenesis for coronary artery disease. *Ann Intern Med* 2002; **136**: 54–71.

31 Vasa M, Fichtlscherer S, Adler K, et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation* 2001; **103**: 2885–90.