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Detection of Grb-2-related Adaptor Protein Gene (*GRAP*) and Peptide Molecule in Salivary Glands of MRL/*lpr* Mice and Patients with Sjögren's Syndrome

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The pathogenesis of Sjögren's syndrome (SS) is poorly understood. In this study we used an in-house mouse spleen cDNA microarray to analyse genes in spleens from MRL/*lpr* (an SS mouse model) mice. We have previously demonstrated that *GRAP* genes were up-regulated in salivary glands of the same mice. The microarray analysis showed that seven out of 2304 genes were highly expressed in spleens from the MRL/*lpr* mice, one of which was the *GRAP* gene. In other words, the *GRAP* gene is highly expressed in the salivary glands and spleen of MRL/*lpr* mice. We also carried out immunohistochemical studies. Mouse and human Grb-2-related adaptor protein (*GRAP*) antigens were expressed on ductal cells and infiltrating lymphocytes in salivary glands of MRL/*lpr* mice and SS patients, but only weakly in controls (MRL/+ mice and individuals with salivary cysts). These results suggest that the *GRAP* gene might have a role in the pathogenesis of SS.

KEY WORDS: SJÖGREN'S SYNDROME; MRL/*lpr* MICE; IN-HOUSE cDNA MICROARRAY; *GRAP* GENE

Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized by progressive lymphocytic infiltration and destruction of exocrine glands, such as the salivary and lacrimal glands. The lymphocytic infiltrate contains predominantly CD4+ T-cells and some B-cells including plasma cells. Dryness of the eyes and mouth is the most typical feature. It either occurs as an isolated disorder (primary form), or more often in association with another autoimmune disease (secondary form).[1–4]

The mechanism of onset and progression of

SS has been poorly understood. It has been proposed that a combination of immunological, genetic and environmental factors may play an important role in the aetiology of SS, but there remains considerable controversy. Many attempts have been made to investigate different aspects of SS. The animal model is one of the most effective approaches to studying the pathogenesis of SS, and several mouse models, such as NFS/*sld*[5,6] and MRL/*lpr* mice,[7-9] have been generated.

The MRL/*lpr* mouse is the classic murine model of autoimmune disorders, and spontaneously develops massive lymphadenopathy, arthritis, vasculitis and nephritis. It develops a disease serologically, pathologically and symptomatically similar to human SS. Significant inflammatory changes develop in the salivary glands between 12 and 16 weeks of age.[7-9] MRL/*lpr* mice have a defect in the *Fas* antigen gene which codes a critical molecule mediating apoptosis.

To understand the mechanism of onset and progression of SS, it is necessary to identify SS-related genes. To clarify the heterogeneity of gene expression in patients with SS, we began by investigating genes up-regulated in the SS mouse model.[10] We demonstrated increased expression of two genes – *IL-16* and *GRAP* – in the salivary glands of MRL/*lpr* mice using an in-house mouse spleen cDNA microarray.[10] The aim of the present study was to investigate gene expression in the spleen of the SS mouse model using a spleen cDNA microarray generated in-house. We also studied the tissue localization (using immunohistochemical staining) of Grb-2-related adaptor protein (GRAP) molecules in specimens obtained from the SS mouse model, and a human homologue of GRAP in specimens from patients with SS.

The *GRAP* genes are apoptosis-related genes, and their possible role in the pathogenesis of organ-specific autoimmune lesions in SS is discussed in light of the findings of our study.

Subjects and methods

SJÖGREN'S SYNDROME MODEL MICE

Sixteen-week-old MRL/MpJ-*lpr/lpr* (MRL/*lpr*) female mice (a model mouse for SS) and 16-week-old MRL/MpJ-+/+ (MRL/+) female congenic control mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan). They were kept under standard conditions, according to the guidelines adopted by the Centre for Animal Experimentation, Nihon University School of Medicine, for 1 week and killed by cervical dislocation. The spleens were quickly removed, frozen in liquid nitrogen, and stored at –80 °C until use.

The ethics review committees for animal experimentation of the participating institutions approved the experimental protocol.

PREPARATION OF mRNA

Total RNA was prepared from spleens of all mice using a TRIzol® reagent (Life Technologies, Rockville, MD, USA). Subsequent cleanup was carried out using an RNeasy Maxi kit® (Qiagen, Germany) according to the manufacturer's instructions. mRNA was obtained from the total RNA using an Oligotex-dT30 mRNA purification kit® (TaKaRa Shuzo Co., Kyoto, Japan). The purified mRNA was subjected to our routine quality control procedure using formaldehyde agarose gels.

PREPARATION OF THE cDNA MICROARRAY

We constructed a cDNA microarray of mouse spleen chips in-house. It consisted of 2304 cDNAs derived from a mouse spleen library (2256 clones). This library was subjected to the oligo-capping method and cDNAs were prepared as described previously.[11,12]

Briefly, 2 mg/ml of polymerase chain reaction products, obtained by amplifying

the spleen library using universal primers, was mixed in a 1:1 ratio with 4 mg/ml nitrocellulose on dimethyl sulphoxide (DMSO)-coated glass slides (Nisshinbo, Chiba, Japan) using a robotic (SPBIO-2000®, Hitachi Software Engineering Co., Yokohama, Japan). This array includes cDNAs of housekeeping genes, such as human β-actin and glyceraldehyde-3-phosphate dehydrogenase, as internal controls, the *luciferase* gene from *Photinus pyralis* as a positive control, and human Cot I DNA as a negative control.[13]

MICROARRAY PROCEDURES

The microarray was hybridized with cDNA probes prepared from a 2-mg mRNA sample from the salivary glands or spleen of diseased and their congenic mice, and labelled with Cy3 fluorochrome (red) and Cy5 fluorochrome (green), respectively. cDNA probes were mixed, applied to the cDNA microarray, and incubated at 65 °C overnight in humid conditions. The fluorescent signals from the hybridized microarray were scanned with a fluorescent laser confocal slide scanner (ScanArray 4000®, GSI Lumonics, Ottawa, Canada). Background subtraction and normalization of the results of all arrayed genes were carried out in each spot using the QuantArray® cDNAmicroarray analysis software software (GSI Lumonics, Ottawa, Canada).

Genes showing a high fluorescence intensity (over 1.5 fold compared with control) in MRL/*lpr* mice were considered to be differentially expressed genes. The DNA sequences from the positive spots were reanalysed to confirm their accordance with our library database.

CRITERIA FOR SELECTING SJÖGREN'S SYNDROME-RELATED GENES

We selected the candidate genes that appeared more than four times in six microarray hybridization analyses. Genes that showed a high fluorescence intensity (over 1.5 fold compared with control) in MRL/*lpr* mice were considered as SS-related genes.

COLLECTION OF HUMAN SPECIMENS

Patients with SS and individuals with salivary cysts, recruited from Nihon University Hospital, participated in this study. All patients fulfilled the criteria for a diagnosis of SS as defined by the European Community Study Group on Diagnostic Criteria for Sjogren's Syndrome as a Preparatory Activity sponsored by the Directorate General for Science, Research and Development of the Commission of European Community.[14] The minor labial submucosal salivary gland specimens were taken from the lower lip. Informed consent was obtained prior to taking the biopsy.

IMMUNOHISTOCHEMICAL STAINING

Mouse specimens

Organs removed from the mice were fixed with 10% phosphate-buffered formalin and embedded in paraffin. Mouse salivary gland sections were fixed in 50% acetone at 4 °C for 30 s, then in 100% acetone at 4 °C for 5 min. Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in Tris-buffered saline (TBS; 5 mM Tris–HCl, pH 7.5, 145 mM NaCl) for 5 min and non-specific staining was blocked by incubation with 10% normal mouse serum for 10 min at room temperature. The rabbit affinity purified immunoglobulin G anti-mouse GRAP peptide antibody, which was made specially for this study by Tanpaku Seisei Co. (Takasaki, Japan), was used diluted 1:100 as a primary antibody. The sections were incubated with the diluted primary antibody overnight at 4 °C, rinsed in TBS for 10 min, and incubated

with EnVision® (Peroxidase Rabbit, DAKO, Denmark) for 30 min. This was followed by additional rinsing in TBS, and incubation with a solution containing 10 mg of 3-amino-9-ethyl-carbazol in 50 ml of 0.02 M sodium acetate (pH 5.5) for 10 min and 4 ml of 30% H_2O_2 for 15 min. The sections were then rinsed in TBS, counterstained with Mayer's haematoxylin for 1 min, and mounted with an aqueous mounting medium.

Human specimens

Human salivary gland sections were processed as described for the mouse specimens, except that 10% normal pig serum was used for blocking non-specific staining. The rabbit affinity purified immunoglobulin G anti-human GRAP peptide antibody was prepared (Tanpaku Seisei Co., Takasaki, Japan) and used as described above.

Results

Five patients with SS and four with salivary cysts were involved in the study. Three MRL/*lpr* mice and three MRL/+ mice were also used.

cDNA MICROARRAY ANALYSIS

To analyse the overall gene expression in SS, we carried out cDNA microarray analysis. We identified seven SS-related genes in the spleens of MRL/*lpr* mice compared with those of MRL/+ mice using the mouse spleen cDNA microarray chip (Table 1).[11]

IMMUNOHISTOCHEMICAL STAINING

The *GRAP* gene showed increased expression in spleens of mice by spleen microarray analysis. Immunohistochemical staining revealed substantial differences between the SS model and control mice in the expression of mouse GRAP (Fig. 1).

Immunohistochemical staining of specimens from patients with SS and individuals with salivary cysts indicated that the human homologue of GRAP was expressed on ductal cells and on certain infiltrating cells in the SS patients, but only weakly in the controls (Fig. 2).

Discussion

Sjögren's syndrome is an autoimmune disease characterized by massive infiltration of lymphocytes into exocrine glands such as salivary and lacrimal glands, and the

TABLE 1
The seven genes with increased levels of expression in the spleen of Sjögren's syndrome (SS) model mice identified by cDNA microarray analysis

Accession No.	Gene	Ratio[a]
U88682	Mouse anti-DNA antibody heavy chain variable region mRNA	2.88
XM_134565	Mouse similar to Gag-Pol polyprotein mRNA	1.93
M16072	Mouse Ig active gamma-2a H-chain V-Dsp2.2-J2-C mRNA	1.64
BC036286	Mouse myeloid/lymphoid or mixed-lineage leukaemia 5, mRNA	2.17
NM_025408	Mouse phytoceramidase, alkaline mRNA	3.23
X76772	Mouse mRNA for ribosomal protein S3	1.56
NM_027817	Mouse GRB2-related adaptor protein (*GRAP*), mRNA	2.82

[a]The averages of fold change based on the normalized microarray fluorescent data of diseased (SS model) mice compared with control mice ($n = 6$).

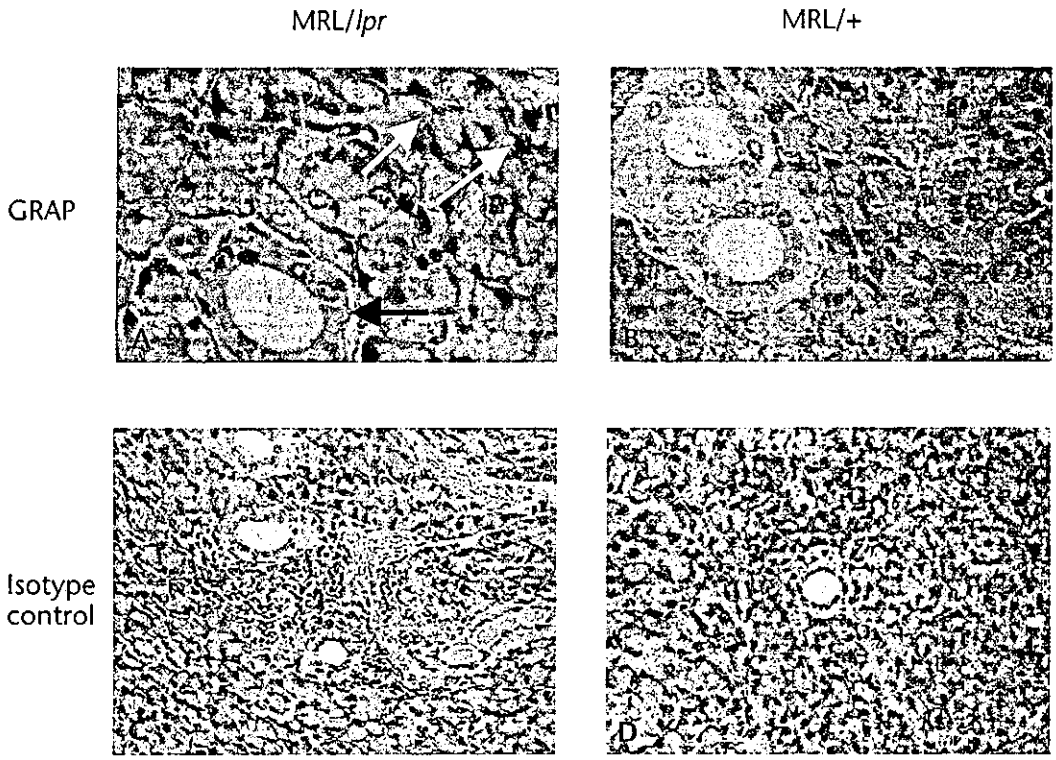

FIGURE 1: Immunohistochemically stained sections from salivary glands of MRL/*lpr* (A) and MRL/+ (B) mice. Expression of GRAP is shown in red. A substantial difference in staining intensity between the diseased (MRL/*lpr*) mice and controls (MRL/+) could be observed. The black arrow indicates ductal cells; white arrows indicate infiltrating cells. The specificity of the GRAP antibody was confirmed by normal rabbit serum immunoglobulin G, an irrelevant antibody (C, D)

subsequent destruction of these glands. Like other autoimmune diseases, the aetiology of SS remains unclear, but previous studies suggest the involvement of hereditary and environmental factors in the onset and progression of the disease. The disease is usually benign and many patients have a normal lifespan. The most common symptoms, dry eyes and dry mouth, however, are problematic and profoundly influence quality of life. In addition to these relatively benign manifestations, abnormalities of more vital organs such as renal tubular acidosis, interstitial pulmonary fibrosis and central nervous system involvement have been demonstrated.[1-4] It is therefore of importance to determine the causes of SS for better disease management.

The animal model is one of the most useful tools in the study of the pathogenesis of SS. The MRL/*lpr* mouse, a mouse model for SS, carries the *lpr* genetic defect, a mutation of the *Fas* gene, and spontaneously develops general lymphadenopathy, glomerulonephritis, systemic vasculitis and sialadenitis.[7-9] These mice are characterized by the presence of high amounts of circulating autoantibodies, such as rheumatoid factor, anti-dsDNA antibodies and immune complexes reminiscent of human systemic lupus erythematosus and rheumatoid arthritis. The onset and extent of disease in these mice are

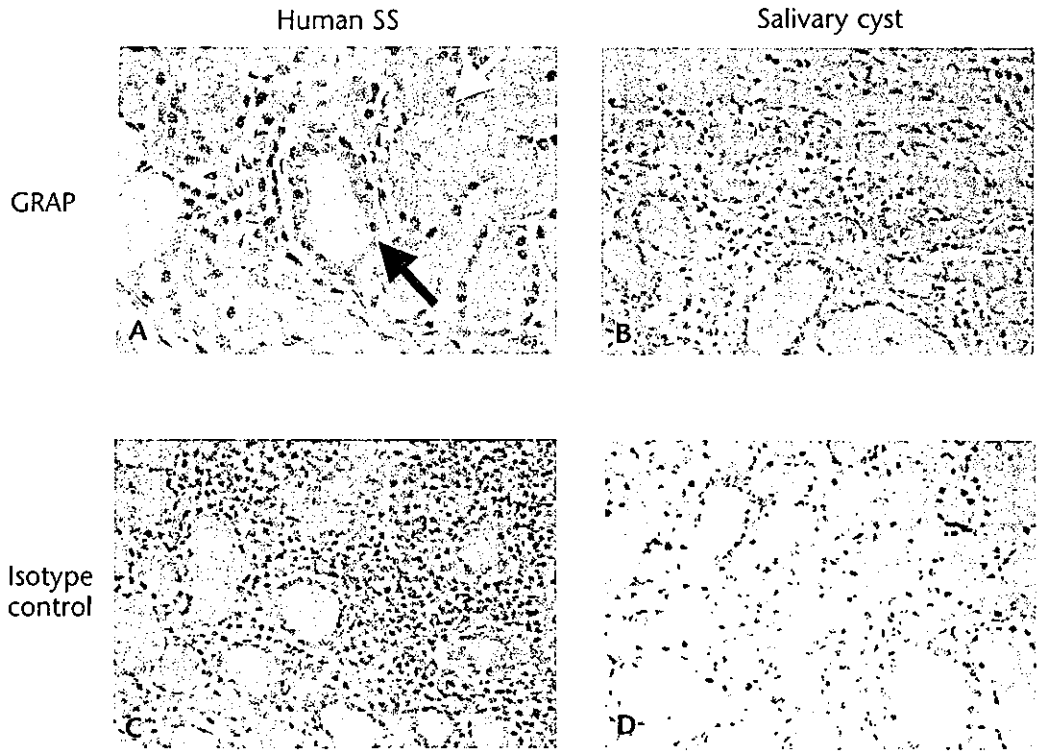

FIGURE 2: Immunohistochemically stained sections from labial salivary gland specimens obtained from patients with Sjögren's syndrome (SS) (A) and those with salivary cysts (B). Expression of GRAP is shown in red. A substantial difference in staining intensity between specimens from SS patients and salivary cysts can be seen. The black arrow indicates ductal cells; the white arrow indicates infiltrating cells. The specificity of the GRAP antibody was confirmed by normal rabbit serum immunoglobulin G, an irrelevant antibody (C, D)

also influenced by other genes, even if the basic genetic abnormality depends on the *lpr* mutation.

To identify SS-related genes, we constructed in-house cDNA microarrays based on mouse spleen cDNA libraries, and carried out cDNA microarray analysis using MRL/*lpr* mice. Up-regulation of *GRAP* genes was identified in the SS mouse model by the mouse spleen cDNA microarray analysis. The *GRAP* gene may contribute to the development of symptoms or the progression of SS.

In the mouse spleen cDNA microarray, three *Fas*-related genes (*DAXX*, *Fas*-antigen and *Fas*-associated protein) were mounted. In the present study, the expression of these three genes (*DAXX* gene, accession no. AF100956; mouse *Fas*-antigen mRNA, accession no. M83649; and mouse Mort 1 *Fas*-associated protein mRNA, accession no. M83649) was almost the same in MRL/*lpr* and MRL/+ mice. In addition, the mouse spleen cDNA microarray includes 10 genes that are obviously linked to T-cells. These genes tend to be highly expressed in the MRL/*lpr* mice compared with the MRL/+ mice. They did not strictly fulfil our criteria, thus we could not consider these as up-regulated genes.

GRAP is one of the adaptor molecules which effectively deliver signals from the immune cell surface to a down-stream functional molecule. GRAP has a structural arrangement of an SH3–SH2–SH3 domain, which is similar to other

immune cell adaptor molecules such as Grb2, GADS and GRAP2.[15,16,17] GRAP is known to be specifically expressed in lymphoid tissues, and structurally resembles Grb2 more than other Grb2 family molecules in that GRAP does not have the proline-rich motif. By immune cell activation, GRAP binds to phosphorylated tyrosine of the local area transport (LAT) at its SH2 region, and further binds to *Son of sevenless* in a way similar to Grb2. Further downstream events remain unknown. In our study, expression of the *GRAP* gene in the salivary glands of the SS mice was higher than that of the control mice. These results suggest that in diseased salivary glands and spleen, enhanced stimulation of the T-cell receptor augments signal transduction to downstream molecules associated with apoptosis. Further detailed analysis of the Grb2 family may clarify the regulation of T-cell differentiation and apoptosis in SS.

Our study provides a strategy to sort out genes that are linked to cell death or T-cell differentiation in SS using the cDNA microarray system. Moreover, our study demonstrates that the *GRAP* gene might be one of the most important SS-related genes participating in the predation and digestion of dead cells after apoptosis. In addition to the cDNA microarray analysis, we also carried out immunohistochemical analysis and demonstrated increased GRAP protein expression in the salivary glands from patients with SS. We found that GRAP was expressed on ductal cells and some infiltrating cells. Because GRAP is an apoptosis-related molecule, GRAP up-regulation might be found in the ductal cells and some infiltrating cells where apoptosis was seen to be present.

These findings also suggest that another pathway for apoptosis, such as the mitogen-activated protein kinase-mediated pathway, exists in SS besides the *Fas/Fas* ligand pathway.

Most recently, Winer *et al.*[18] reported a new autoantigen, ICA69, that may play an important role in the progression of the disease in another primary SS mouse model – the non-obese diabetic mouse. ICA69 cDNA was not mounted on the cDNA chip in our study, but it would be of interest to examine the expression of the *ICA69* gene in MRL/*lpr* mice using our microarray system. Further analysis of the candidate genes identified in this study and SS-related molecules like ICA69 will help clarify the pathogenesis of SS.

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References

1 Talal N, Sokoloff L, Barth WF: Extrasalivary lymphoid abnormalities in Sjögren's syndrome (reticulum cell sarcoma, 'pseudolymphoma', macroglobulinemia). *Am J Med* 1967; 43: 50 – 65.
2 Hoffman RW, Alspaugh MA, Waggie KS, Durham JB, Walker SE: Sjögren's syndrome in MRL/*lpr* and MRL/n mice. *Arthritis Rheum* 1984; 27: 157 – 165.
3 Moutsopoulous HM, Fauci AS: Immunoregulation in Sjögren's syndrome: influence of serum factors on T-cell subpopulations. *J Clin Invest* 1980; 65: 519 – 528.
4 Fox RI: Clinical feature, pathogenesis, and treatment of Sjögren's syndrome. *Curr Opin Rheumatol* 1996; 8: 438 – 445.
5 Haneji N, Hamano H, Yanagi K, Hayashi Y: A new animal model for primary Sjögren's syndrome in NFS/sld mutant mice. *J Immunol* 1994; 153: 2769 – 2777.

6 Hayashi Y, Haneji N, Hamano H, Yanagi K, Takahashi M, Ishimaru N: Effector mechanism of experimental autoimmune sialadenitis in the mouse model for primary Sjögren's syndrome. *Cell Immunol* 1996; 171: 217 – 225.
7 Nose M, Nishimura M, Ito MR, Toh J, Shibata T, Sugisaki T: Arthritis in a novel congenic strain of mice derived from MRL/*lpr* lupus mice: genetic dissociation from glomerulonephritis and limited autoantibody production. *Am J Pathol* 1996; **149**: 1763 – 1769.
8 Ito MR, Terasaki S, Itoh J, Katoh H, Yonehara S, Nose M: Rheumatic diseases in an MRL strain of mice with a deficit in the functional Fas ligand. *Arthritis Rheum* 1997; **40**: 1054 – 1063.
9 Shiraiwa H, Takei M, Yoshikawa T, Azuma T, Kato M, Mitamura K, *et al*: Gene expression analysis in the exocrine glands of the NSF/sld and the MRL/*lpr* mice, animal models for primary and secondary Sjögren's syndrome, using a cDNA microarray. *Arthritis Rheum* 2001; 9 (Suppl): S251.
10 Wang Y, Nose M, Kamoto T, Nishimura M, Hiai H: Host modifier genes affect mouse autoimmunity induced by the *lpr* gene. *Am J Pathol* 1997; **151**: 1791 – 1798.
11 Azuma T, Takei M, Yoshikawa T, Nagasugi Y, Kato M, Shiraiwa H, *et al*: Identification of candidate genes for Sjögren's syndrome using MRL/*lpr* mouse model of Sjögren's syndrome and cDNA microarray analysis. *Immunol Lett* 2002; **81**: 171 – 176.
12 Yoshikawa T, Nagasugi Y, Azuma T, Kato M, Sugano S, Hashimoto K, *et al*: Isolation of novel mouse genes differentially expressed in brain using cDNA microarray. *Biochem Biophys Res Commun* 2000; **275**: 532 – 537.
13 Otsuka M, Aizaki H, Kato N, Suzuki T, Miyamura T, Omata M, *et al*: Differential cellular gene expression induced by hepatitis B and C viruses. *Biochem Biophys Res Commun* 2003; **300**: 443 – 447.
14 Vitali C, Bombardieri S, Moutsopoulos HM, Balestrieri G, Bencivelli W, Bernstein RM, *et al*: Preliminary criteria for the classification of Sjogren's syndrome. Results of a prospective concerted action supported by the European Community. *Arthritis Rheum* 1993; **36**: 340 – 347.
15 Ma W, Xia C, Ling P, Qiu M, Luo Y, Tan TH, *et al*: Leukocyte-specific adaptor protein GRAP2 interacts with hematopoietic progenitor kinase 1 (HPK1) to activate JNK signaling pathway in T lymphocytes. *Oncogene* 2001; **20**: 1703 – 1714.
16 Liu SK, Berry DM, McGlade CJ: The role of Gads in hematopoietic cell signaling. *Oncogene* 2001; **20**: 6284 – 6290.
17 Gong Q, Cheng AM, Akk A, Alberola-Ila J, Gong G, Pawson T, *et al*: Disruption of T cell signaling networks and development by Grb2 haploid insufficiency. *Nat Immunol* 2001; **2**: 29 – 36.
18 Winer S, Astsaturov I, Cheung R, Tsui H, Song A, Gaedigk R, *et al*: Primary Sjögren's syndrome and deficiency of ICA69. *Lancet* 2002; 9339: 1063 – 1069.

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A New Tactile Skin Sensor for Measuring Skin Hardness in Patients with Systemic Sclerosis and Autoimmune Raynaud's Phenomenon

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We used a new tactile sensor to measure the elastic properties of skin in patients with systemic sclerosis or Raynaud's phenomenon. The sensor consists of a piezoelectric vibrator with vibration pickup to measure frequency changes when the sensor is placed on the skin. The mean frequency change at the skin surface of the proximal third phalanx in patients with systemic sclerosis was significantly lower than in age- and sex-matched controls. The results in systemic sclerosis patients were statistically correlated to the Modified Rodnan Skin Thickness Score. This technique was also used to measure the therapeutic efficacy of salpogrelate, a new specific serotonin receptor antagonist. A greater mean frequency change was seen after treatment. We conclude that this new tactile sensor is useful for quantitatively measuring skin sclerosis and may help determine the efficacy of therapeutic treatments.

KEYWORDS: SKIN SENSOR; SKIN HARDNESS; SYSTEMIC SCLEROSIS; RAYNAUD'S PHENOMENON

Introduction

A number of studies have been published on the use of instruments for investigating the elastic properties of human skin,[1 - 6] and such instruments have facilitated many advances in the fields of dermatology and cosmetic science. In general, the ground substance in skin[7] shows thixotropic behaviour, that is it shows a reduction in viscosity as shear stress increases, and the decreased viscosity is recovered slowly on standing. For example, Finlay,[8] using a rotary displacement servo system, showed that human skin contains a thixotropic ground substance that enables gliding of the outer surface in a complex pattern. The viscoelastic and complex biomechanical structure of skin necessitates the use of simplified mechanical models to measure and assess its biomechanical properties.[9] Developing a tactile sensor and measuring system allowed detection of the stiffness- and elasticity-related properties of human skin.[10]

The sensor is based on a piezoelectric element and can measure displacement. In addition, it is hand-held and designed for easy and convenient use.

In patients with systemic sclerosis (SSc) the biomechanical properties of skin, such as elasticity and extensibility, are altered by fibrosis. We investigated whether this new tactile sensor is a useful tool for measuring skin hardness in patients with SSc, Raynaud's phenomenon or mixed connective tissue disease (MCTD; patients with MCTD usually have scleroderma and Raynaud's disease). The results were compared with those of the gold standard Modified Rodnan Skin Thickness Score (MRSTS). In addition, we investigated the use of the tactile sensor to measure the efficacy of salpogrelate treatment.

Patients and methods

PATIENTS

Systemic sclerosis patients who fulfilled the American College of Rheumatology (formerly the American Rheumatism Association) classification criteria[11] and patients with Raynaud's phenomenon diagnosed on the basis of characteristic triphasic digital colour changes[12] were included in the study. The SSc patients had suffered from diffuse atrophic or sclerotic skin changes for at least 5 years. Confirmation of the diagnosis of SSc was obtained from a forearm skin biopsy. Patients with MCTD were also investigated and the diagnosis was based on a high serum titre of anti-U1 ribonuclease-sensitive extractable nuclear antigen antibodies. The clinical features of MCTD were an overlap of diffuse connective tissue diseases (systemic lupus erythematosus, SSc and polymyositis).

Age- and sex-matched controls were also recruited; these included patients with other autoimmune diseases (except for MCTD) and healthy individuals without Raynaud's phenomenon.

Those enrolled in the study had no other skin disorders, did not use vibrating tools, and did not participate in any sport that affected the skin on their fingers. Informed consent was obtained from all the participants, but ethical approval was not sought.

THE TACTILE SENSOR

The tactile sensor consists of a piezoelectric vibrator (61 kHz) with vibration pickup to measure the change in frequency when the sensor is placed on the skin; it is connected to a computer equipped with the appropriate software. Integrated with the tactile sensor is a displacement sensor that shows the spring compression loading when the sensor element is placed against the skin during measurement. Under certain conditions (e.g. fixed contact pressure), the change in frequency reflects the acoustic impedance of the object and is related to the stiffness of the soft tissue.

MEASUREMENT OF SKIN HARDNESS

Briefly, the measurements were made with the tactile sensor oscillating at a resonance frequency of about 61 kHz. It was set to measure the change in resonance frequency (ΔF). $\Delta F = f_x - f_0$, where f_x is the resonance frequency of the sensor placed on the skin and f_0 is the non-contact frequency (61 kHz). A total of 150 measurements were made in sequence. To compare the degree of skin sclerosis quantitatively, the mean ΔF value between the 100th and 110th detection points, when the measurement became stable, was used.

Since the contact area of the sensor tip depends on the contact pressure, the resonance frequency of the piezoelectric element also changes according to how hard

the sensor tip is pressed against the surface of the skin. The contact pressure of the sensor element was held constant by the spring. The compression of the spring when the sensor probe touched the skin (and thus the movement of the sensor element) was continually measured by the displacement transducer.

The ΔF was measured at the skin surface of the proximal third phalanx. The mean ΔF was also measured at the locations assessed when calculating the MRSTS.[13-15]

MODIFIED RODNAN SKIN THICKNESS SCORE

The MRSTS was assessed by clinical palpation at multiple locations, assigning a score of 0 – 3 to each site (0, normal; 1, thickened; 2, thickened, unable to pinch; 3, thickened, unable to move).[13-15]

MEASUREMENT OF TREATMENT EFFICACY

The change in ΔF was measured in patients with autoimmune Raynaud's phenomenon, SSc or MCTD after administering the serotonin (5-hydroxytryptamine) receptor antagonist salpogrelate[16] at a dose of 300 mg/day for 4 weeks. Measurements were taken in the same way as described in the section on measuring skin hardness before and after salpogrelate therapy.

Salpogrelate therapy is used routinely in the clinic as therapy for these conditions.

STATISTICAL ANALYSIS

Results are given as the mean ± SD. Student's *t*- and *F*-tests were used for statistical comparison. A *P*-value < 0.05 was considered statistically significant. Statistical calculations were performed using the statistical software package STATVIEW II (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Five patients with SSc, 13 with Raynaud's phenomenon without SSc and MCTD, and five patients with MCTD were recruited to the study. There were a total of 23 age- and sex-matched controls, all of whom were female and ranged in age from 44 years to 66 years (mean ± SD 47.1 ± 15.8 years). The control women comprising six patients with systemic lupus erythematosus, three with Sjögren's syndrome, three with anti-phospholipid antibody syndrome, four with rheumatoid arthritis, two with Behçet's disease and five healthy individuals without Raynaud's phenomenon.

SKIN HARDNESS

The mean ΔF at the skin surface of the proximal third phalanx in patients with either atrophic or sclerotic SSc was significantly lower than in the age- and sex-matched controls (Table 1). The mean ΔF values at the locations used to calculate the MRSTS are shown in Table 2. MRSTS values were calculated for four patients, three with and one without SSc.

CORRELATION BETWEEN TACTILE SENSOR MEASUREMENTS AND MRSTS

To investigate the relationship between our measurements of skin hardness (using the tactile sensor) and the MRSTS, comparisons were made between MRSTS and mean ΔF values. Initial experiments demonstrated that the mean ΔF values associated with an MRSTS of 2 or 3 were statistically different from those associated with an MRSTS of 0 ($P < 0.001$). The mean ΔF value associated with an MRSTS of 1, however, was not statistically different from that associated with an MRSTS of 0 (Fig. 1).

MEASURING TREATMENT EFFICACY

We tested whether or not the sensor is useful for measuring treatment efficacy. The changes in ΔF measured after treating nine

TABLE 1
Demographic data of patients and changes in frequency (ΔF) measured using the tactile sensor in patients with systemic sclerosis (SSc) ($n = 15$), Raynaud's phenomenon ($n = 13$), mixed connective tissue disease (MCTD, $n = 5$), and age- and sex-matched controls ($n = 23$) for healthy individuals without Raynaud's phenomenon

Skin condition	Age (years)	Mean age (years)	Anti-Scl-70 antibody	Intraphalangeal ΔF (Hz)	Mean ± SD intraphalangeal ΔF (Hz)	Finger tip ΔF (Hz)	Mean ± SD Finger tip ΔF (Hz)
SSc	49			1167		1331	
SSc	62			1039		1016	
SSc	62		Positive	2148		779	
SSc	41		Positive	1585		1945	
SSc	45		Positive	1302		79	
		51.8			1448 ± 441[a]		1030 ± 771[a]
MCTD	30			1882		2853	
MCTD	66			2103		1507	
MCTD	67			2162		1502	
MCTD	43			2122		2415	
MCTD	28			2456		3055	
		46.8			2145 ± 205[a]		2266 ± 733
Raynaud's phenomenon	49				2070 ± 229[a] ($n = 13$)		2847 ± 315 ($n = 7$)*
Controls	55				2575 ± 199 ($n = 23$)		2712 ± 437 ($n = 8$)*

[a] $P < 0.005$ compared with the mean control value (Student's *t*-test).
*Finger tip ΔF was only measured in some patients with Raynaud's phenomenon and some controls.

TABLE 2
Mean (±SD) change in frequency (ΔF) measured by the tactile sensor and Modified Rodnan Skin Thickness Scores (MRSTS) at multiple locations in patients with systemic sclerosis (SSc) and one control person

Location	SSc1 (diffuse type)		SSc2 (diffuse type)		SSc3 (limited type)		Non-SSc*	
	Mean ± SD ΔF (Hz)	MRSTS	Mean ± SD ΔF (Hz)	MRSTS	Mean ± SD ΔF (Hz)	MRSTS	Mean ± SD ΔF (Hz)	MRSTS
Finger								
Right	1479 ± 14	3	1475 ± 7.5	3	1754 ± 20	3	2282 ± 5	0
Left	1431 ± 11	3	1613 ±10	3	1618 ± 25	3	2344 ± 4	0
Hand								
Right	2089 ± 12	3	1582 ± 5.3	3	1582 ± 5	3	2217 ± 6	0
Left	1965 ± 15	3	1911 ± 3.7	3	1613 ± 10	3	2469 ± 5	0
Forearm								
Right	2270 ± 15	1	2084 ± 5.2	2	ND	2	2464 ± 50	0
Left	2329 ± 6	1	1691 ± 9	3	ND	2	2170 ± 11	0
Upper arm								
Right	2033 ± 3	2	2027 ± 1.6	3	2170 ± 11	2	2354 ± 6	0
Left	1944 ± 4	2	1885 ± 4.6	3	2186 ± 6.8	2	2234 ± 5	0
Anterior chest	1584 ± 33	3	2196 ± 5.1	1	2186 ± 6.8	0	2186 ± 7	0
Face (cheek)								
Right	1711 ± 32	3	1262 ± 9.1	3	2360 ± 7.9	3	2359 ± 8	0
Left	1893 ± 5	3	ND	ND	ND	3	2422 ± 4	0
Abdomen	1845 ± 14	3	1990 ± 14	3	2110 ± 1.6	2	2110 ± 2	0

TABLE 2 (continued)
Mean (± SD) change in frequency (ΔF) as measured by the tactile sensor and Modified Rodnan Skin Tightness Score (MRSTS) at multiple locations in three patients with systemic sclerosis (SSc) and one control person

Location	SSc1 (diffuse type)		SSc2 (diffuse type)		SSc3 (limited type)		Non-SSc*	
	Mean ± SD ΔF (Hz)	MRSTS	Mean ± SD ΔF (Hz)	MRSTS	Mean ± SD ΔF (Hz)	MRSTS	Mean ± SD ΔF (Hz)	MRSTS
Thigh								
Right	1786 ± 18	3	1792 ± 1.3	3	2218 ± 6.5	0	ND	ND
Left	1683 ± 43	3	2021 ± 2	3	2469 ± 5.3	0	ND	ND
Leg								
Right	1976 ± 7	3	2083 ± 1.7	2	ND	0	ND	ND
Left	1822 ± 6	3	1709 ± 4.3	3	1789 ± 4.4	0	ND	ND
Foot								
Right	1842 ± 5	3	1742 ± 1.7	3	2021 ± 6.5	1	2193 ± 5	0
Left	1922 ± 8	3	1911 ± 3.7	3	2193 ± 5.1	1	2248 ± 20	0

ND, not done.
*The non-SSc patient (control) had interstitial pneumonia and a low Scl-70 serum titre.

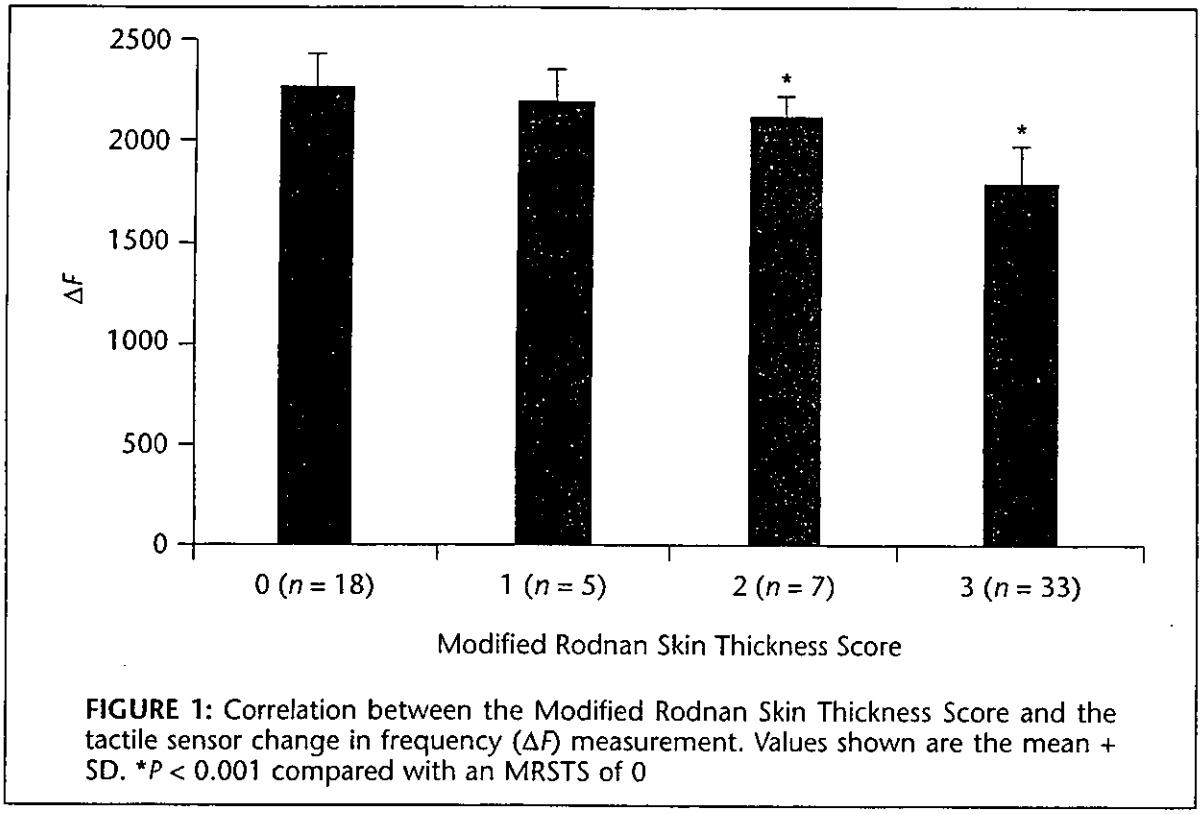

FIGURE 1: Correlation between the Modified Rodnan Skin Thickness Score and the tactile sensor change in frequency (ΔF) measurement. Values shown are the mean + SD. *$P < 0.001$ compared with an MRSTS of 0

patients (six with Raynaud's phenomenon, one with SSc and two with MCTD) with salpogrelate for 4 weeks are shown in Table 3.

In patient R2K, who suffered from unclassified connective tissue disease, ΔF was improved more (from 2040 to 2418) when treated with prostaglandin ointment and salpogrelate than with salpogrelate alone. In patient R6N who suffered from SLE, ΔF was not changed after taking low dose prednisone (from 1875 to 1822), but improved after taking salpogrelate and prednisolone (from 1822 to 2286). In this pilot study, salpogrelate improved the ΔF in the patients tested.

Discussion

There are several scores that use pinching to measure skin thickness, including the MRSTS, which is currently the gold standard. This test has been proven to be reasonably reliable,[14,17,18] but inexperienced general physicians may find it difficult to use. We report here a novel, non-invasive tactile sensor[19] that assesses physical properties such as stiffness and inverse compliance by measuring the change in resonance frequency when a vibrating ceramic rod touches the surface of an object. Preliminary results on measuring the physical properties of living tissue such as skin have given promising results.[20,21] It has been evaluated both in a standardized silicone gum model and in a rat testis model, and was compared with an impression method that measures interstitial pressure and water displacement in skin.[22] The two methods correlated well, and the tactile sensor was able to detect differences that correlated with silicone stiffness as measured by an international standard (DIN ISO 2137, 150 g hollow cone). We have further developed this tactile sensor and measuring system to detect the stiffness- and elasticity-related properties of human skin. The sensor is hand-held and designed for easy and convenient use. Experimental

TABLE 3
Change in frequency (ΔF) as measured by the tactile sensor in patients with autoimmune Raynaud's phenomenon, systemic sclerosis (SSc) or mixed connective tissue disease (MCTD), before and after treatment with salpogrelate (300 mg/day for 4 weeks)

Patient	Age (y)	Diagnosis	ΔF (Hz)		Other therapy
			Before salpogrelate	After salpogrelate	
R1K	42	UCTD, AP	2176	2447	Aspirin
R2N	65	UCTD, AP	1620	2418	Prostaglandin ointment (ΔF = 2040 with salpogrelate alone)
R3A	58	IP, Scl-70	2367	2976	Prednisone
R4T	23	SLE	1727	2263	Aspirin
R5C	45	RA	2133	2647	Prednisone
R6N	34	SLE, AIHA	1822	2286	Prednisone (ΔF = 1875 with prednisolone alone)
P1H	41	SSc, Scl-70	1585	1960	None
M1H	43	MCTD	2122	3029	None
M2M	28	MCTD	2456	2768	Prednisone

UCTD, unclassified connective tissue disease; AP, anti-phospholipid antibody; IP, interstitial pneumonia; Scl-70, anti-Scl-70 antibody; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; AIHA, autoimmune haemolytic anaemia.

results on silicone gum and in healthy Japanese and Swedish women indicated that the instrument was able to detect changes in the stiffness- and elasticity-related properties of human skin related to age, day-to-day variations and the application of cosmetics.[10]

In the atrophic phase an SSc patient's skin becomes sclerotic and, as a result, its elasticity and viscosity decrease. When we used this new tactile sensor to quantitatively measure sclerosis of the skin, it was shown to be very sensitive in detecting changes in skin properties in SSc patients. In addition, the results using this new method were statistically correlated to the gold standard MRSTS. We also found some decrease in ΔF in patients with autoimmune Raynaud's phenomenon. The reason for this was unclear, since histopathological data on the skin in these patients were not available. It has been suggested that autoimmune Raynaud's phenomenon may represent a pre-SSc state,[23] and that recurrent Raynaud's phenomenon results in fibrotic change in the small vessels. It is also possible that the tactile sensor might be influenced by the skin blood flow.

To investigate whether this new tactile sensor might be helpful in determining the efficacy of treatment, we used it to measure changes in the ΔF after treatment with

salpogrelate. The efficacy of this new specific serotonin receptor antagonist has been reported in Japanese patients with Raynaud's phenomenon.[24] In contrast, another serotonin antagonist, ketanserin, failed to show efficacy compared with placebo in a North American trial.[25] Salpogrelate and ketanserin have been shown to produce similar effects on serotonin-induced platelet aggregation and DNA synthesis of aortic smooth muscle cells due to their antagonism of 5-hydroxytryptamine-2 receptors.[26,27] Salpogrelate, however, does not exhibit an α_1-adrenergic receptor blocking action, unlike ketanserin,[28] and it is possible that the biological responses and side-effects of salpogrelate and ketanserin are not the same.

Our study shows that this new tactile sensor might be useful for quantitatively measuring sclerosis of the skin and assessing therapy efficacy. Further experiments are necessary to determine the efficacy of the sensor in relation to various other pathological states.

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References

1 Berardesca E, Gabba P, Farinelli N, Borroni G, Rabbiosi G: Skin extensibility time in women. Changes in relation to sex hormones. *Acta Derm Venereol* 1989; **69**: 431 – 433.
2 Escoffier C, de Rigal J, Rochefort A, Vasselet R, Leveque J-L, Agache PG: Age-related mechanical properties of human skin: an *in vivo* study. *J Invest Dermatol* 1989; **93**: 353 – 357.
3 Maes D, Short J, Turek BA, Reinstein JA: *In vivo* measuring of skin softness using the gas bearing electrodynamometer. *Int J Cosmet Sci* 1983; **5**: 189 – 200.
4 Marchdahlgen R, Elsnau WH: Measurement of skin condition by sonic velocity. *J Soc Cosmet Chem* 1984; **35**: 1 – 19.
5 Fthenakis CG, Maes DH, Smith WP: *In vivo* assessment of skin elasticity using ballistrometry. *J Soc Cosmet Chem* 1991; **42**: 211 – 222.
6 Omata S, Asano H, Ozaki S: A new type tactile sensor for detecting the hardness of objects like the human hand. *Tissue Eng* 1989; **14**: 99 – 102.
7 Brace RA, Guyton AC: Interstitial fluid pressure: capsule, free fluid, gel fluid, and gel absorption pressure in subcutaneous tissue. *Microvasc Res* 1979; **18**: 217 – 228.
8 Finlay JB: Thixotropy in human skin. *J Biomech* 1978; **11**: 333 – 342.
9 Fung YC: Mechanical properties of living tissues. In: *Biomechanics* (Fung YC, ed). New York: Springer-Verlag, 1981; pp1 – 584.
10 Lindahl OA, Omata S, Angquist KA: A tactile sensor for detection of physical properties of human skin *in vivo*. *J Med Eng Technol* 1998; **22**: 147 – 153.
11 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 – 590.
12 Leroy EC, Medsger TA, Jr: Raynaud's phenomenon: a proposal for classification. *Clin Exp Rheumatol* 1992; **10**: 485 – 488.
13 Rodnan GP, Lipinski E, Luksick J: Skin thickness and collagen content in progressive systemic sclerosis and localized scleroderma. *Arthritis Rheum* 1979; **22**: 130 – 140.
14 Black CM: Measurement of skin involvement in scleroderma. *J Rheumatol* 1995; **22**: 1217 – 1219.
15 Clements PJ: Measuring disease activity and severity in scleroderma. *Curr Opin Rheumatol* 1995; **7**: 517 – 521.
16 Kikumoto R, Hara H, Ninomiya K, Osakabe M, Sugano M, Fukami H, *et al*: Syntheses and platelet aggregation inhibitory and antithrombotic properties of [2-[(ω-aminoalkoxy) phenyl]ethyl]benzenes. *J Med Chem* 1990; **33**: 1818 – 1823.
17 Clements PJ, Lachenbruch PA, Ng SC, Simmons M, Sterz M, Furst DE: Skin score. A semiquantitative measure of cutaneous involvement that improves prediction of prognosis in systemic sclerosis. *Arthritis Rheum* 1990; **33**: 1256 – 1263.
18 Brennan P, Silman A, Black C, Bernstein R, Coppock J, Maddison P, *et al*: Reliability of skin involvement measures in scleroderma. The UK Scleroderma Study Group. *Br J Rheumatol* 1992; **31**: 457 – 460.
19 Omata S, Terunuma Y: New tactile sensor like the human hand and its applications. *Sens Actuators A Phys* 1992; **35**: 9 – 15.
20 Omata S: New type tactile sensor for sensing hardness like the human hand and its applications for living tissue. Technical Digest,

9th Sensory Symposium, Tokyo, Japan, 30 – 31 March 1990; pp257 – 260.
21 Omata S, Terunuma Y: Measurement of skin elasticity using new type tactile sensor and application to acupuncture therapy. Proceedings of the 3rd USA–China–Japan Conference on Biomechanics, 1991, August 25 – 29, Georgia Institute of Technology, Atlanta, Georgia, USA, 1991; pp153 – 154.
22 Lindahl OA, Omata S: Impression technique for the assessment of oedema: comparison with a new tactile sensor that measures physical properties of tissue. *Med Biol Eng Comput* 1995; 33: 27 – 32.
23 Black CM: The aetiopathogenesis of systemic sclerosis: thick skin – thin hypotheses. The Parkes Weber Lecture 1994. *J R Coll Physicians Lond* 1995; 29: 119 – 130.
24 Kumagai S, Morinobu A, Ozaki S, Nakao K, Ishida H: Sarpogrelate hydrochloride for Raynaud's phenomenon of patients with collagen diseases (in Japanese, English abstract). *Ryumachi* 1998; 38: 504 – 510.
25 Seibold J: Prospective, controlled trial of ketanserin vs placebo in systemic sclerosis (SSc). *Arthritis Rheum* 1995; 38: S1091.
26 Pietraszek MH, Takada Y, Taminato A, Yoshimi T, Watanabe I, Takada A: The effect of MCI-9042 on serotonin-induced platelet aggregation in type 2 diabetes mellitus. *Thromb Res* 1993; 70: 131 – 138.
27 Sharma SK, Zahradka P, Chapman D, Kumamoto H, Takeda N, Dhalla NS: Inhibition of serotonin-induced vascular smooth muscle cell proliferation by sarpogrelate. *J Pharmacol Exp Ther* 1999; 290: 1475 – 1481.
28 Maruyama K, Kinami J, Sugita Y, Takada Y, Sugiyama E, Tsuchihashi H, *et al*: MCI-9042: high affinity for serotonergic receptors as assessed by radioligand binding assay. *J Pharmacobiodyn* 1991; 14: 177 – 181.

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Early Bone Marrow Hematopoietic Defect in Simian/Human Immunodeficiency Virus C2/1-Infected Macaques and Relevance to Advance of Disease

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To clarify hematological abnormalities following infection with human immunodeficiency virus (HIV), we examined the hematopoietic capability of bone marrow by using cynomolgus monkeys infected with pathogenic simian/human immunodeficiency virus (SHIV) strain C2/1, an animal model of HIV infection. The relationship between the progress of the infection and the CD4/CD8 ratio of T lymphocytes or the amount of SHIV C2/1 viral load in the peripheral blood was also investigated. A colony assay was performed to assess the hematopoietic capability of bone marrow stem cells during the early and advanced phases of the infection. Colonies of granulocytes-macrophages (GM) were examined by PCR for the presence of the SIVmac239 *gag* region to reveal direct viral infection. There was a remarkable decrease in the CFU-GM growth on days 1 and 3 postinoculation, followed by recovery on day 56. During the more advanced stage, the CFU-GM growth decreased again. There was minimal evidence of direct viral infection of pooled cultured CFU-GM despite the continuously low CD4/CD8 ratios. These results indicate that the decrease in colony formation by bone marrow stem cells is reversible and fluctuates with the advance of the disease. This decrease was not due to direct viral infection of CFU-GM. Our data may support the concept that, in the early phase, production of inhibitory factors or deficiency of a stimulatory cytokine is responsible for some of the bone marrow defects described in the SHIV C2/1 model.

It is generally known as a feature of human immunodeficiency virus (HIV) infection that CD4-positive T lymphocytes and monocytes infected with pathogenic HIV or simian/human immunodeficiency virus (SHIV) decrease in number and disappear. Infected hosts will thus become immunodeficient. Moreover, it has been reported that, after HIV infection is contracted, hematological abnormalities in the bone marrow and the peripheral blood such as anemia, lymphopenia, and thrombocytopenia ensue and correlate with the advance of the illness (36). Several possibilities have been noted as the cause of such hematological abnormalities: the apoptosis of virus-infected cells, changes in the hematological environment, dysfunction of the thymus or the lymphoid system, change of cell division, or dysfunction of hematopoietic progenitor cells (1). Furthermore, a few reports have shown that the bone marrow of patients with AIDS displays morphological alterations similar to those of patients with myelodysplastic syndrome (2, 31). The term "HIV myelopathy" has been used for this bone marrow pathology by some investigators (10, 22).

Reduced numbers of CFU (burst-forming units–erythrocytes [BFU-E] or CFU–granulocytes-macrophages [CFU-GM]) have been reported in bone marrow samples from patients infected with HIV (9, 16, 27). Moreover, the reduction in CFU-GM resembles that of an animal model of AIDS experimentally induced by simian immunodeficiency virus (SIV) (13, 30, 32). While the precise mechanisms of such hematopoietic abnormalities remain unclear, several hypotheses have been proposed: (i) decreased levels of appropriate cytokines secondary to altered numbers of T-cell subsets or macrophages, which are commonly seen in HIV type 1 (HIV-1) infection (28); (ii) production of inhibitory factors (14, 29); (iii) cytotoxic elimination of the precursor cells by the antibody-dependent cell-mediated cytolytic mechanism (7); and (iv) infection of hematopoietic precursor cells with viruses, which leads to death of these cells or their metabolic alteration (7). On the other hand, it has been suggested that primitive bone marrow progenitor cells are most likely not a major reservoir for HIVs (6, 13, 28).

Despite mounting data supporting the above-mentioned hypotheses, a unifying explanation remains elusive. We studied bone marrow samples from cynomolgus monkeys (*Macaca fascicularis*) experimentally infected with an SHIV strain in order to evaluate possible cellular and molecular events that affect hematopoiesis in SHIV infection.

MATERIALS AND METHODS

Animals. Twenty cynomolgus monkeys (nine males and 11 females) used in this study were maintained in our facility according to the guidelines of the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases (NIID), Tokyo, Japan. All treatments were performed according to the standard operating procedures for monkeys for evaluation of human vaccines in the Tsukuba Primate Center, NIID, Tsukuba, Ibaraki, Japan. Their ages were 3 to 6 years, and their weights were approximately 3 to 5 kg (Table 1). Four sham-inoculated monkeys were included as a control. They were inoculated with saline alone instead of virus-containing saline solution. Two additional monkeys without sham treatment also served as a negative control. Low-dose ketamine (intramuscular dose of 10 mg/kg of body weight) was used as an anesthetic for blood and bone marrow sampling.

Viruses. A highly pathogenic SHIV strain, designated C2/1, was obtained by serum passages in cynomolgus monkeys. The SHIV C2/1 strain contains the *env*

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TABLE 1. Protocol for control and infection of cynomolgus monkeys with SHIV C2/1 and subsequent bone marrow harvesting[a]

Monkey no.	Age (yr)	Sex	Day of bone marrow harvesting	Virus administration	Dose of inoculated virus ($TCID_{50}$)
13	5	Male			
44	4	Male			
181	6	Male			
1037	5	Male			
1091	5	Male			
759	4	Male			
4345	4	Male	1	Intravenous	20
1	5	Female	3	Intravenous	20
2	5	Female	3	Intravenous	20
90c	5	Female	56	Intravenous	20
560	4	Female	56	Intrarectal	2,000
430	4	Female	56	Intrarectal	2,000
442	3	Female	56	Intrarectal	2,000
200	5	Female	56	Intravenous	20
944	5	Male	56	Intravenous	20
520	5	Female	56	Intrarectal	20
844	4	Female	56	Intrarectal	20
0634	4	Female	56	Intravenous	10
054	4	Female	113	Intravenous	2,000
039	4	Male	380	Intravenous	20

[a] Monkeys 13 to 759 were controls that were not infected with SHIV. Monkeys 4345 to 039 were inoculated intravenously or intrarectally with the doses of SHIV C2/1 shown in the table. Bone marrow harvesting was performed on the indicated day after inoculation. $TCID_{50}$, 50% tissue culture infective dose.

gene of pathogenic HIV-1 strain 89.6. This chimeric virus was propagated in concanavalin A-activated peripheral blood mononuclear cells (PBMC) from healthy monkeys or in a cell line, M8166. Cell-free virus stocks were stored at −120°C (25).

Antibodies. The mouse monoclonal antibodies (MAbs) used in this study were fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated MAbs to monkey CD3 (NF-18; BioSource) and to human CD4 (Nu-T H/I; Nichirei), CD8 (Nu-T S/C; Nichirei), CD16 (3G8; Pharmingen), and CD20 (Leu-16; Becton Dickinson).

Preparation of bone marrow cells. Fourteen monkeys were infected with SHIV C2/1 at three 50% tissue culture infective doses by intravenous or intrarectal inoculation (Table 1). Bone marrow samples were aspirated from their femoral bone during autopsy. For a sham-inoculated control, monkeys received 0.5 ml of saline alone. One day or 3 days later, bone marrow samples were aspirated from their pelvic bones. Non-sham-control monkeys received only ketamine anesthesia for bone marrow aspiration.

Preparation of blood samples for cell surface antigen analysis by flow cytometry. Peripheral blood was mixed with lysis buffer (Becton Dickinson) and centrifuged at 300 × *g* for 5 min. Viable cells were counted by the trypan blue dye-exclusion method. The cell surface antigens CD3, CD4, CD8, CD16, and CD20 were stained with their respective MAbs. After being washed with staining buffer, 5×10^4 cells in each labeled sample tube were analyzed by a FACSCalibur flow cytometer (Becton Dickinson) with use of Cell Quest software (Becton Dickinson). Absolute PBMC count was determined as follows. Fifty milliliters of each whole-blood sample, containing FITC-conjugated anti-CD3 MAb (BioSource), PE-conjugated anti-CD4 MAb (Becton Dickinson), and peridinin-chlorophyll protein-conjugated anti-CD8 MAb (Becton Dickinson), was added to a TRUCOUNT tube and incubated at room temperature. Contaminating red blood cells were lysed, and each sample was analyzed by flow cytometry as described above. All measurements were made under the same instrumental setting.

Quantification of cell-associated and plasma viral load. Plasma viral RNA was extracted and purified using a QIAamp viral RNA minikit (Qiagen, Valencia, Calif.). For quantitative analysis of the RNA, reverse transcriptase-PCR (RT-PCR) was performed with primers and probes targeting the SIVmac239 *gag* region, designed by computer with the Primer Express software (PE Biosystems). The viral RNA was reverse transcribed and amplified using a Taqman EZ RT-PCR kit (PE Biosystems) with the designed primers (forward primer, 5′-AATGCAGAGCCCCAAGAAGAC-3′, and reverse primer, 5′-GGACCAAGGCCTAAAAAACCC-3′) and detected with a probe, FAM-5′-ACCATGTTATGGCCAAATGCCCAGAC-3′-TAMRA. Probed products were quantitatively monitored by their fluorescence intensity with ABI 7700 (PE Biosystems). For a positive-control RNA, SIVmac239 *gag* RNA was synthesized and purified using a MEGAscript kit (Ambion, Austin, Tex.) with template plasmid pKS460. This template contained the SIVmac239 *gag* sequence within the T7 promoter region. Plasma viral load, measured in duplicate, was estimated based on a standard curve of the control RNA and the RNA recovery rate (19).

Performance of colony assays on bone marrow specimens and detection of the SIVmac *gag* sequence by PCR in pooled cultured CFU-GM. Bone marrow samples (n = 20) were obtained by aspiration from the femoral or pelvic bones of monkeys. An approximately 10-ml bone marrow sample diluted with phosphate-buffered saline was slowly layered on top of 10 ml of sterile Ficoll-Hypaque in a 15-ml conical tube. The tubes were then centrifuged at 400 × *g* for 30 min at room temperature. With use of a pipette, a top plasma layer was removed, and a mononuclear cell layer was transferred in a small volume to a tube. After two washes with 2% fetal calf serum–Iscove's medium (code no. HBM-3160; Stem Cell Technologies Inc.), cell density was adjusted to 10^6 mononuclear cells/ml. The cell suspensions were then mixed with methylcellulose medium (Methocult HF4434; Stem Cell Technologies) so that it gave a final concentration of 10^5 cells per 1.1 ml for final plating. The cell culture was performed in duplicate in 35-mm-diameter plastic dishes at 37°C, 5% CO_2, and 100% humidity for 10 days, and colonies (BFU-E, CFU-GM, and CFU-granulocytes-erythroids-macrophages-megakaryocytes) were counted by inverted microscopy. CFU-GM were plucked from the methylcellulose culture and collected in pools and then subjected to PCR analysis by the method described above.

RESULTS

Figure 1 shows the relationship between the CD4/CD8 ratios of the peripheral blood T cells of infected monkeys and the postinoculation time. In general, the CD4/CD8 ratio decreased in 14 to 21 days after inoculation. It has been reported that monkeys inoculated with SHIV C2/1 had transient decreases of $CD4^+$ T lymphocytes within several days after infection (25). In this study, one monkey showed a decrease in CD4/CD8 ratio even within several hours: namely, the CD4/CD8 ratio of monkey 4345 decreased to 1.28 in 6 h after inoculation and went up to 1.80 in 24 h (Fig. 1). Control monkeys showed only negligible declines (Fig. 1).

The number of viral copies was estimated for four animals (200, 944, 520, and 844) by real-time PCR (Fig. 2). It has been

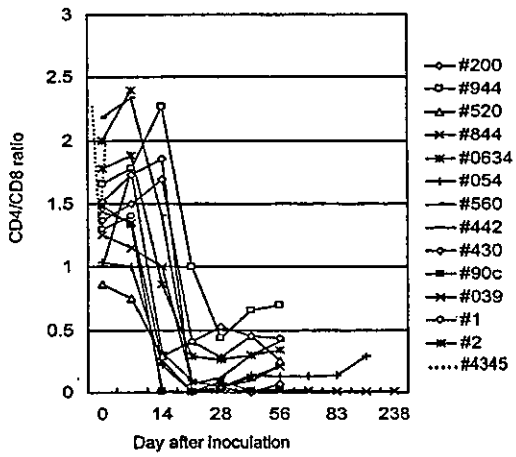

FIG. 1. Changes of CD4/CD8 ratio in monkeys inoculated with SHIV C2/1. All monkeys showed decreased CD4/CD8 ratios between day 14 and day 21 after inoculation. Monkey 4345 had a decrease in CD4/CD8 ratio in the first 24 h. Control monkeys showed only negligible declines in the first 24 h (preinoculation, 1.26 ± 0.400 [mean ± standard deviation]; 6 h, 1.24 ± 0.259; 24 h, 1.11 ± 0.323; n = 4).