

Combining effects of polymorphism of tumor necrosis factor α 5'-flanking region and HLA-DRB1 on radiological progression in patients with rheumatoid arthritis

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Abstract We examined whether polymorphisms upstream of the TNF- α gene (*TNFA*) were associated with the radiological progression of rheumatoid arthritis (RA). One hundred and twenty-three patients with early RA (disease duration <1 year) were enrolled in a prospective follow-up study. The laboratory findings (ESR, CRP, and RF) were evaluated every 2 months for 2 years. Radiological progression in hands/wrists and feet was evaluated every 6 months for 2 years using Larsen's score. *HLA-DRB1* genotype was determined by PCR-RFLP method. The genotypes for -1031, -863, and -857 single-nucleotide polymorphisms in the upstream 5'-flanking region of *TNFA*

were determined by a PCR-preferential homoduplex formation assay in patients with RA and 265 healthy controls. Four *TNFA* alleles (U01, U02, U03, and U04) were identified. The frequency of individuals with U02 was significantly higher in patients than in controls ($P = 0.0025$). Radiographs of hands/wrists/feet were available for 72 patients after 1 year and for 73 patients after 2 years. When the *HLA-DRB1* genotype was analyzed simultaneously, patients possessing U02 without an *HLA-DRB1* shared epitope (SE) (U02+SE-) showed the lowest progression of Larsen's score (12 months). There was no difference in the level of ESR, CRP, or RF at the first visit among U02+SE+, U02+SE-, U02-SE+, and U02-SE- groups. The combination of the polymorphism of the *TNFA* upstream promoter region and *HLA-DRB1* allele was associated with radiological progression in the early stage of RA.

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease, and its etiology is unknown. *HLA-DRB1* alleles encoding the shared epitope (SE) are associated with RA. On the other hand, a variety of cytokines have been detected in the synovial fluid and synovial tissue of patients with RA. TNF- α , a proinflammatory cytokine, plays a pivotal role in RA [1]. TNF- α is produced mainly by monocytes and macrophages, but also by B cells, T cells, and fibroblasts. Transgenic mice that overexpress the human TNF gene develop polyarthritis similar to RA [2]. Moreover, significant clinical benefit has been observed in RA patients who received TNF blocking agents.

The human TNF- α gene (*TNFA*) is located on chromosome 6 within the MHC class III region between HLA-B and HLA-DR genes. A number of polymorphisms have been identified in the *TNFA* itself, and a polymorphic allele of the *TNFA* may correlate with a particular clinical subtype of disease. More recently, it has been reported that *TNFA*-308 polymorphisms, but not SE alleles are associated with increased radiological progression [3], although *TNFA*-308 polymorphisms are relatively rare in Japanese [4]. On the other hand, Higuchi et al. [4] identified three new polymorphisms, -1031T/C, -863C/A, and -857T/C, in the upstream 5'-flanking region of *TNFA* in Japanese. Seki et al. and Shibue et al. [5, 6] reported that the frequency of the -857T allele in RA patients is significantly higher than in controls, although *HLA-DRB1* is more strongly associated with disease susceptibility than *TNFA* polymorphisms.

In this study, we determined polymorphisms of the 5'-flanking region of *TNFA* in Japanese patients with early RA to further examine whether the polymorphisms were associated with susceptibility to and progression of RA.

Patients and methods

The patients were derived from those reported in our previous study [7]; thus, they visited our outpatient clinic at the Institute of Rheumatology, Tokyo Women's Medical University, within 1 year of symptom onset and were enrolled in a prospective follow-up study between 1991 and 1995. For this study, we used 123 of 198 patients, who satisfied the American College of Rheumatology (ACR) 1987 criteria for RA [8]. All patients were Japanese. Twenty-five patients were males, and 98 patients were females. All patients were treated with disease-modifying antirheumatic drugs and/or nonsteroidal anti-inflammatory agents selected by rheumatologists in our outpatient clinic. No patients were treated with anti-TNF- α therapy, because this study was performed before anti-TNF- α therapy was introduced into Japan. We used the same population of patients as reported recently [9]. Controls consisted of 265 healthy Japanese volunteers with no inflammatory rheumatic diseases. One hundred forty-five were males, and 120 were females.

The radiological progression of hands/wrists and feet was evaluated every 6 months for 2 years using Larsen's score. The radiographs were read randomly by three rheumatologists. The sum of the Larsen grades for two wrists, eight finger proximal interphalangeal (PIP) joints, two thumb interphalangeal (IP) joints, ten metacarpophalangeal (MCP) joints, eight metatarsophalangeal (MTP) joints of toes 2-5, and the great toe IP joints was used to determine the overall joint damage score on a scale of

0-160. We defined the progressive score as the Δ damage score: the damage score at the first visit subtracted from the damage score at 12 or 24 months. All participants gave informed consent.

Genomic DNA was extracted from peripheral blood leukocytes using the standard phenol-chloroform extraction procedure. The *HLA-DRB1* genotype was determined using the polymerase chain reaction (PCR)-restriction fragment length polymorphism method. Alleles considered to have SE were *DRB1* *0101, *0401, *0404, *0405, *0410, and *1001. Genotyping of *TNFA* -1031, -863, and -857 single-nucleotide polymorphisms (SNPs) was carried out using a PCR-preferential homoduplex formation assay [10]. Four haplotypes, -1031/-863/-857 TCC, TCT, CAC, and CCC, were designated as *TNFA*-U01, -U02, -U03, and -U04, respectively. The laboratory variables, ESR (mm/h, Westergren method), serum CRP (mg/dl), and rheumatoid factor (RF) were measured in each patient every 2 months for 2 years. RF status was assessed using a particle agglutination test (RAPA test; Fujizoki Pharmaceutical, Tokyo, Japan).

Statistical significance of the differences between groups was examined by Fisher's exact test or by the Mann-Whitney *U* test (Statview; Abacus Concepts Inc., Berkeley, CA). *P*-values less than 0.05 were regarded as significant, and all tests were two-tailed. As for the comparison of the Δ damage score, the Bonferroni procedure was used to control the type I error rate. We reported the unadjusted *P* values.

Results

The characteristics of the patients at study entry are summarized in Table 1. The mean age of all patients was 49.5 years, and disease duration averaged 5.8 months. Most patients (87%) were RF positive.

As shown in Table 2, a significant positive association with RA was observed for *DRB1* *0405 (*P* = 0.00012). On

Table 1 Characteristics of 123 patients with RA

Variables	<i>n</i>
Female/male	98/25
Age (years)	49.5 \pm 12.3
Disease duration (months)	5.8 \pm 3.2
Positive RAPA	107 (87%)
ESR (mm/h)	48.8 \pm 24.7
CRP (mg/dl)	2.3 \pm 3.2

Quantitative data are shown as the mean \pm SD unless otherwise noted

ESR erythrocyte sedimentation rate, CRP C-reactive protein

Table 2 Carrier frequency of *HLA-DRB1* alleles among RA patients and controls

<i>DRB1</i> allele	RA (%) (<i>n</i> = 123)	Control (%) (<i>n</i> = 265)	OR	<i>P</i> -value
0101	19 (15.4)	26 (9.8)	1.68	NS
0401	6 (4.9)	6 (2.3)	2.21	NS
0403	5 (4.1)	13 (4.9)	0.82	NS
0404	2 (1.6)	4 (1.5)	1.08	NS
0405	54 (43.9)	65 (24.5)	2.41	0.00012*
0406	8 (6.5)	19 (7.2)	0.90	NS
0410	3 (2.4)	5 (1.9)	1.30	NS
1001	1 (0.8)	2 (0.8)	1.08	NS
1101	5 (4.1)	5 (1.9)	2.20	NS
1302	11 (8.9)	51 (19.2)	0.41	0.0099*
1405	5 (4.1)	17 (6.4)	0.62	NS
1406	2 (1.6)	7 (2.6)	0.61	NS
SE (+)	78 (63.4)	100 (37.7)	2.86	2.3×10^{-6} *

Values are the number (%) of patients; *P*-value was determined by Chi-square analysis

RA rheumatoid arthritis, NS not significant

* Statistically significant

Table 3 Analysis of *TNFA* promoter region haplotype in RA patients and controls

	RA (%) (<i>n</i> = 123)	Control (%) (<i>n</i> = 265)	OR	<i>P</i> -value
Haplotype positivity				
<i>TNFA</i> -U01	108 (88)	232 (88)	1.02	0.94
<i>TNFA</i> -U02	58 (47)	83 (31)	1.95	0.0025*
<i>TNFA</i> -U03	20 (16)	69 (26)	0.55	0.033*
<i>TNFA</i> -U04	2 (2)	14 (5)	0.30	0.158
Haplotype frequency (<i>n</i> = 246) (RA) / (<i>n</i> = 530) (Control)				
<i>TNFA</i> -U01	160 (65)	349 (66)	0.964	0.825
<i>TNFA</i> -U02	64 (26)	94 (18)	1.63	0.008
<i>TNFA</i> -U03	20 (8)	73 (14)	0.554	0.024
<i>TNFA</i> -U04	2 (1)	14 (3)	0.302	0.095
Allele frequency				
-1031 T	224 (91)	443 (84)		
C	22 (9)	87 (16)		
-863 C	226 (92)	457 (86)		
A	20 (8)	73 (14)		
-857 C	182 (74)	436 (82)		
T	64 (26)	94 (18)		

* Statistically significant

the other hand, the frequency of individuals with *DRB1* *1302 was significantly lower in patients than in controls ($P = 0.0099$). The frequency of individuals with SE was significantly higher in patients than in controls ($P < 0.0001$) (Table 2).

The proportion of individuals with the *TNFA*-U02 allele was significantly higher in patients than in controls ($P = 0.0025$). The proportion of individuals with U03 was significantly lower in patients than in controls ($P = 0.033$) (Table 3).

We divided patients into four groups, depending on the positivity of U02 and SE alleles: U02+SE+, U02+SE-, U02-SE+, and U02-SE-. The frequency of the genotype combination U02+SE+ was significantly higher in patients than in controls ($P = 0.0013$) (Table 4). The frequency of

U02-SE+ was also significantly higher in RA than in controls, but the frequency of U02+SE- was not different between RA and controls.

Matsushita et al. [10] reported that *TNFA*-U02 is in linkage disequilibrium with *DRB1* *0405. Thus, we examined linkage disequilibrium between *TNFA* alleles and *HLA-DRB1* alleles using LDSUPPORT [11] and also found that *TNFA*-U02 was in strong linkage disequilibrium with *HLA-DRB1* *0405 (data not shown).

Radiographs of hands/wrists/feet were available for 72 patients after 1 year and for 73 patients after 2 years. The X-ray progression score (Δ damage score) at 12 months was slightly higher in patients with SE than in patients without SE ($P = 0.0444$) (Fig. 1a). Although the progression score (Δ damage score) at 12 months was lower in the

Table 4 *TNFA*-U02 haplotype and *HLA-DRB1* SE allele distribution among RA patients and controls

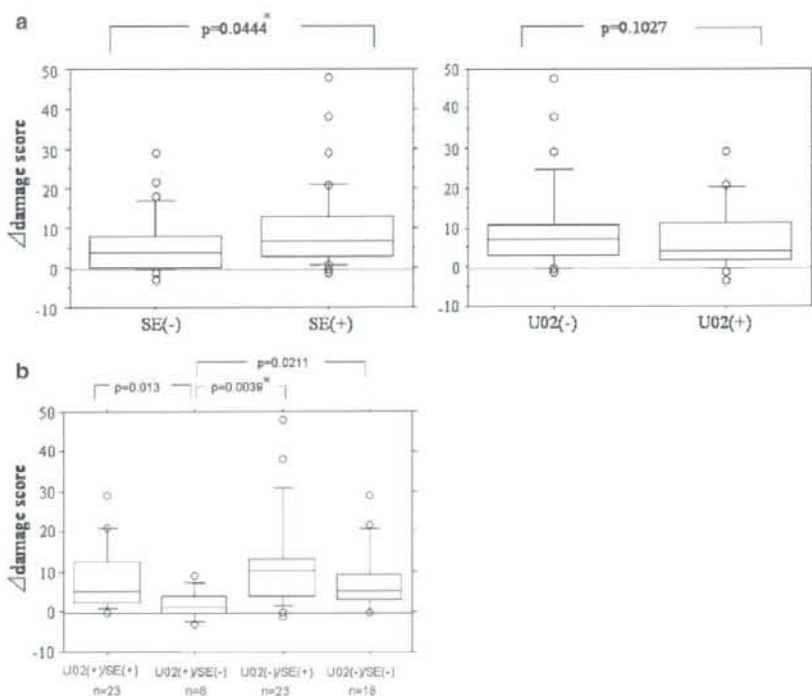
	RA (%) (n = 123)	Control (%) (n = 265)	OR	P-value
U02 (+)/SE (+)	41 (33)	49 (18)	2.2	0.0013*
U02 (+)/SE (-)	17 (14)	34 (13)	1.1	0.788
U02 (-)/SE (+)	37 (30)	51 (19)	1.8	0.0177*
U02 (-)/SE (-)	28 (23)	131 (49)	0.3	<0.0001*

Values are the number (%) of patients; P-value was determined by chi-square analysis

RA rheumatoid arthritis

* Statistically significant

Fig. 1 Progression of damage score (12 months). P value was determined by the Mann-Whitney U test. All values are the mean \pm SEM, or box plots, in which the upper and lower bars show the 90th and 10th percentiles, respectively, and the upper, center, and lower lines of the box show 75th, 50th, and 25th percentiles, respectively. **a** *Statistically significant, **b** *statistically significant using the Bonferroni procedure



U02+ group than in the U02- group, the difference was not statistically significant ($P = 0.1027$). To further examine the role of *TNFA* promoter haplotype in X-ray progression, *HLA-DRB1* SE genotype was analyzed in combination. There was no difference in the levels of ESR, CRP, or RF at the first visit among the U02+SE+, U02+SE-, U02-SE+, and U02-SE- groups (Table 5). As shown in Fig. 1b, patients with U02+SE- showed the lowest progression score at 12 months among the four groups and those with U02-SE+ showed the highest score. Thus, the Δ damage score at 12 months was significantly lower in the U02+SE- group than in the U02-SE+ group using the Bonferroni procedure. The difference in the Δ damage score between the U02-SE- group and U02-SE+ group was not significant. There was no

difference in the percentage of MTX treatment for 2 years among the four groups, although none of the U02+SE- patients received MTX (Table 5); however, these differences were not significant at 24 months (data not shown).

Discussion

In this study, we observed that the association of *TNFA* promoter polymorphism with RA can be explained by linkage disequilibrium between *TNFA* and *HLA-DRB1* alleles, as reported previously, even in patients in the early stages. We also demonstrated that *TNFA* alleles may have some additional effects on *HLA-DRB1* alleles, although the contribution of *TNFA* alleles alone on X-ray progression

Table 5 Laboratory data and MTX treatment

	ESR (mm/h) at first visit	CRP (mg/dl) at first visit	MTX at 12th month	MTX at 24th month
U02 (+)/SE (+)	48.4 ± 22.2	2.2 ± 3.1	1/23	3/26
U02 (+)/SE (-)	55.6 ± 25.3	3.4 ± 3.7	0/8	0/5
U02 (-)/SE (+)	46.9 ± 28.1	2.6 ± 3.9	3/23	3/24
U02 (-)/SE (-)	47.8 ± 23.6	1.7 ± 1.7	1/18	1/18

Quantitative data are shown as the mean ± SD unless otherwise noted
ESR erythrocyte sedimentation rate, CRP C-reactive protein

was not significant. The progression score of U02+SE- patients was significantly lower than the score of U02-SE+. In addition, the progression score of U02+SE- patients was lowest among the four groups at 12 months, although these differences were not significant. On the other hand, the progression score of U02-SE+ was highest among the four groups; however, such tendency was not significant, partly because of the small number of patients ($n = 73$). Further, the difference in the progression score between U02-SE- and U02-SE+ was not significant ($P = 0.198$). We speculate that SE interference was small in the U02- group.

Udalova et al. [12] reported that the TNF-863A allele specifically reduces the binding of NF- κ B p50/p50 homodimer to this part of the TNF promoter region and then increases TNF expression. In addition, they showed that the proportion of individuals with the TNF-863A allele was higher in patients undergoing joint replacement surgery for RA than controls [13]. These results support our findings because the group of patients with U02, that is, without TNF-863A, showed slower progression than the group of patients without U02 (Fig. 1a). On the other hand, the same group showed that TNF-857C homozygotes produce a higher amount of TNF [14]. In addition, Van Krugten et al. [15] showed that RA patients with -857CC genotype tended to develop erosive disease more than -857CT patients. These results support our findings that the U02+ group (without TNF-857C) had slower radiological progression than the U02- group (Fig. 1a). Recently, Kang et al. [16] reported that RA patients with the T allele at position -857 of the *TNFA* promoter responded better to etanercept therapy than C-allele homozygotes, supporting our findings. On the other hand, Higuchi et al. [4] revealed higher transcriptional promoter activities in the -1031C/-863A or -857T allele. Skoog et al. [17] reported, however, that carriers of the -863A allele had significantly lower serum TNF- α levels. The effects of these polymorphisms on TNF- α transcription and production are contradictory.

In this study, we demonstrated that SE-possessing patients were associated with more erosion than patients without SE during the first year of disease (Fig. 1a); however, the association between *HLA-DRB1* alleles and disease severity is controversial. Kaltenhauser et al. [18] examined 87 patients with RA to evaluate prognostic

makers for severe joint erosion. They showed that the presence of the SE+DR4 allele influenced joint destruction. Matthey et al. [19] reported that RF-negative patients carrying an SE allele had higher Larsen scores than RF-negative patients lacking SE. On the other hand, Valenzuela-Castano et al. [20] reported no relationship between SE and the severity of structural joint damage in large joints. In 1997, we demonstrated that SE alleles failed to correlate with more severe disease [7]. In our current study, we used 123 of these 198 patients, showing a contradiction in the results. We speculate that this contradiction was derived from the different study populations; in 1997, we used patients with polyarthritis within 1 year of symptom onset, but in the current study we selected patients who satisfied the ACR 1987 criteria. In addition, different evaluation methods of disease severity were associated with the contradiction; in 1997, we used bone erosion, but in the current study we used the Δ damage score. Thus, further investigations are needed to clarify this contradiction.

Our study is very important, although it was carried out in the 1990s. Most RA patients were treated with traditional DMARDs, but not with biologics, and we therefore investigated RA patients with the classical therapy alone. Thus, it is impossible to carry out a study like ours in 2008.

In conclusion, our study suggests that the combination of the polymorphism of the *TNFA* upstream promoter region and *HLA-DRB1* allele may identify subgroups of patients with early RA related to radiological progression. Further study analyzing a larger number of patients is needed, and such a study may result in the efficient prediction of RA prognosis at an early stage of disease.

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Conservative sequences in 3'UTR of TCR ζ mRNA regulate TCR ζ in SLE T cells

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Abstract

We have demonstrated that T-cell receptor ζ (ζ) mRNA with a 562-bp deleted alternatively spliced 3'-untranslated region (3'UTR) observed in T cells of patients with systemic lupus erythematosus (SLE) can lead to a reduction in ζ and TCR/CD3 (J. Immunol., 2003 & 2005). To determine the region in ζ mRNA 3'UTR for the regulation of ζ , ζ mRNA with 3'UTR truncations ligated into pDON-AI was used to infect murine T-cell hybridoma MA5.8 cells, which do not contain ζ . As a Western blot analysis demonstrated the importance of the regions from +871 to +950, containing conservative sequence 1 (CS1), and +1070 to +1136, containing CS2, for the production of ζ , we constructed MA5.8 mutants carrying ζ mRNA 3'UTR with deletions of these regions (Δ CS1 and Δ CS2 mutants). Western blot and FACS analyses showed significant reduction in the cell surface ζ and TCR/CD3 in both these mutants, and IL-2 production was decreased, compared with MA5.8 cells transfected with wild-type ζ mRNA. Furthermore, real-time PCR demonstrated the instability of ζ mRNA with 3'UTR deletions in these MA5.8 mutants. In conclusion, CS1 and CS2 may be responsible for the regulation of ζ and TCR/CD3 through the stability of ζ mRNA in SLE T cells.

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Keywords: Systemic lupus erythematosus; TCR ζ ; 3'UTR; Signal transduction; Conservative sequence; T-cell receptor; IL-2; Autoimmune disease; MA5.8 cells; mRNA stability

Systemic lupus erythematosus (SLE) is well known as a prototype systemic autoimmune disease [1]. Defects in signal transduction through the TCR/CD3 complex may cause T-cell dysfunction and autoimmunity in NOD mice as well as in MRL *lpr/lpr* mice [2–4]. Several studies, including our own, have identified a functional defect in early signaling molecules on peripheral blood T cells (PBTs) as a cause of T-cell dysfunction in human SLE [5–7]. Sakaguchi et al. [8] demonstrated that a ZAP-70 mutation causes autoimmune arthritis in the SKG mouse model, supporting the notion that functional defects in early signaling molecules can cause autoimmune diseases.

On the other hand, we and other groups have reported that a reduction in tyrosine phosphorylation and the diminished expression of ζ protein play crucial roles in the pathogenesis of SLE [9–13], and that an aberrant form of the ζ mRNA 3'-untranslated region (3'UTR), which is alternatively spliced and 562-bp shorter than the wild-type 3'UTR, is predominantly expressed in SLE T cells (ζ mRNA/as-3'UTR) [14,15] and leads to the up-regulation of several other proteins [16]. An *in vitro* expression analysis of the ζ protein from ζ mRNA/as-3'UTR using MA5.8 cell mutants and a retrovirus system showed that the predominant expression of ζ mRNA/as-3'UTR leads to the down-regulation of not only ζ , but also of other TCR/CD3 components because of the instability of these ζ mRNA splice variant forms [17]. Observations in our study

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[17] and others [18] suggest that the deleted 562-bp portion of the 3'UTR in ζ mRNA/as-3'UTR is critical for ζ mRNA stability. The 3'UTR region of mRNA is known to control the turnover rate of pre-synthesized mRNAs through interactions with *trans*-acting factors by altering mRNA stability and affecting the transportation and localization of mRNA [19–21]. Messenger RNA 3'UTR contains *cis*-acting elements, i.e., adenosine-uridine (AU)-rich elements (AREs), that bind to *trans*-acting proteins and participate in either the stabilization or destabilization of transcripts. Two AREs are located at positions -735 and $+803$ of the ζ mRNA 3'UTR, and both of these AREs are involved in the 562-bp deleted portion of the ζ mRNA/as-3'UTR. Therefore, we investigated which portion, including the AREs in the ζ mRNA 3'UTR, could be responsible for the stability of ζ mRNA and the production of the TCR/CD3 complex, including ζ , using MA5.8 cell mutants and a retrovirus system. In this study, we demonstrated that two conservative sequences (CSs), rather than the AREs, are important for the production of the TCR/CD3 complex, including ζ , by influencing ζ mRNA stability.

Materials and methods

RT-PCR. RT-PCR was performed according to a previously described method [13,22]. Human ζ cDNA with 3'UTR truncations (740, 871, 950, 1070, 1136, 1330, and 1457) were amplified from the PBTs of a normal healthy control subject using primers that were designed as described in Table 1 on line. The primers for amplifying the murine CD3 ϵ and β -actin cDNA were arranged as previously reported [22].

Cell lines and inhibition of RNA synthesis. The MA5.8 cells (lacking endogenous ζ expression) were kindly provided by Dr. Takashi Saito and the RetroPackTMPT67 (BD Biosciences Clontech, Inc., Palo Alto, CA, USA) was used as the dualtropic packaging cell line. Inhibition of RNA synthesis was performed as previously reported [17].

Construction of MA5.8 mutants. MA5.8 mutants were constructed using a previously described method [17,23]. To construct the MA5.8 mutants with ζ mRNA 3'UTR deletions, Fragments A, B, C, and D were amplified from the PBTs of a normal healthy control subject using primers that were designed as described in Table 1 on line. Fragment A and B or Fragment C and D were ligated into SalI-cut pDON-AI and were then transfected into RetroPackTMPT67 cells.

Real-time PCR. The primers and TaqMan probes for human ζ , murine CD3 ϵ , and murine β -actin were designed as previously reported [23]. Amplification and detection of specific products were performed according to a previously described amplification protocol [17]. Standard curves for the quantification of mRNA were established as previously reported [23].

Western blot. Western blot was performed according to a previously described method [9]. The blots were probed with a mouse anti-human ζ mAb (TIA-2) (Coulter Immunology, Hialeah, FL, USA). TIA-2 was visualized using a peroxidase-conjugated anti-mouse IgG (GE Healthcare Bio-Science Corp., Piscataway, NJ, U.S.A.). Biotinylated proteins were detected using streptavidin-peroxidase (Southern Biotechnology Associates, Birmingham, IL, USA). The densities of the specific bands were quantified as index values using the method previously reported [17].

Flow cytometry. The flow cytometric analysis procedure has been previously described [17].

Antibody stimulation and IL-2 quantification. IL-2 quantification was determined by the method described previously [23].

Statistical analysis. Statistical significance was calculated using the Student *t*-test for unpaired data and Statview software (version 4.5;

Abacus, Berkeley, CA, USA). A value of $p < 0.05$ was considered statistically significant.

Results

RT-PCR of ζ mRNA with 3'UTR truncations

To identify the region of the ζ mRNA 3'UTR responsible for the expression of ζ and the TCR/CD3 complex, human ζ cDNA with 3'UTR truncations (740 [605 bp], 871 [736 bp], 950 [815 bp], 1070 [935 bp], 1136 [1001 bp], 1330 [1195 bp], and 1457 [1322 bp]) were amplified from the PBTs of a normal healthy control (Fig. 1 on line).

Construction of MA5.8 mutants with ζ mRNA 3'UTR truncations

ζ cDNA with 3'UTR truncations (740, 871, 950, 1070, 1136, 1330, and 1457) were ligated into pDON-AI, transfected into RetroPackTMPT67 cells, and used to infect MA5.8 cells to construct the MA5.8 mutants 740, 871, 950, 1070, 1136, 1330, and 1457, respectively. Also, WT and AS3'UTR mutants were constructed from full-length wild-type human ζ cDNA and ζ cDNA/as-3'UTR, respectively. The NEG mutant was constructed using pDON-AI without any insert DNA.

ζ protein expression in MA5.8 mutants with ζ mRNA 3'UTR truncations

In a Western blot analysis using an anti-human ζ mAb (TIA-2), the production of the ζ protein by the 740 and 871 mutants was not observed (Fig. 1). The production of ζ protein by the 1136 (15.4 index), 1330 (16.7 index), and 1457 (17.2 index) mutants was almost the same as that of the WT mutant (18.5 index). However, the ζ protein expression of the 950 (11.0 index) and 1070 (10.2 index) mutants was relatively low. Therefore, we concluded that ζ protein expression gaps might exist between the 871 and 950 mutants and between the 1070 and 1136 mutants,

Construction of MA5.8 mutants with ζ mRNA 3'UTR deletions

To determine whether the ζ mRNA 3'UTR regions from $+871$ to $+950$ and/or from $+1070$ to $+1136$ were responsible for the production of ζ protein, we constructed MA5.8 mutants containing ζ mRNA 3'UTR deletions of these two regions. Fragments A (736 bp, $+136$ to $+871$), B (680 bp, $+952$ to $+1631$), C (940 bp, $+136$ to $+1075$), and D (525 bp, $+1107$ to $+1075$) were amplified using RT-PCR (Fig. 2 on line). Then, Fragments A and B and Fragments C and D were ligated into pDON-AI, transfected into RetroPackTMPT67 cells, and used to infect MA5.8 cells for the construction of the mutants. As the regions from -871 to $+950$ and $+1070$ to $+1136$ contain CS1 (5'-CCCUGCC UUGGGCCUCUGGUUGC-3') and CS2 (5'-C

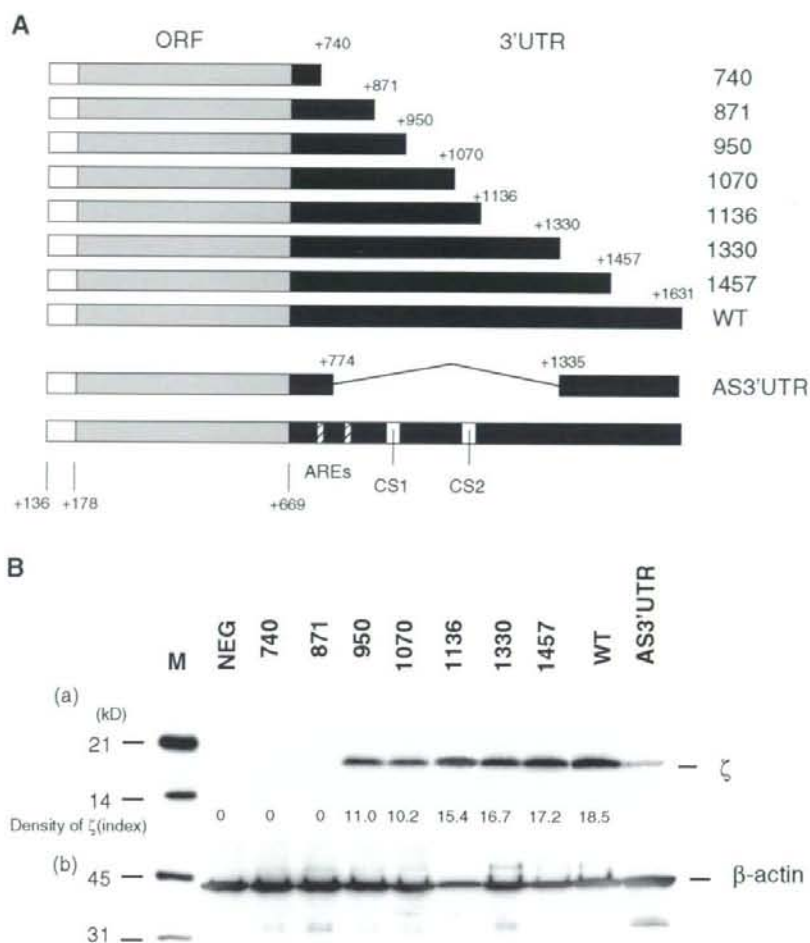


Fig. 1. (A) Scheme of human ζ cDNA with 3'UTR truncations. The shaded bars show the open reading frame (ORF), and the black bars represent the 3'UTR. The white bars show the 5'UTR. AS3'UTR refers to the ζ mRNA/as-3'UTR with a 562-bp deletion (+774 to +1335) in its 3'UTR. The wild-type ζ mRNA 3'UTR contains two A-U rich elements (AREs) (+735 and +803) and two conservative sequences (CSs: CS1, +872 to +951 and CS2, +1076 to +1106). (B) Expression of the ζ protein in MA5.8 mutants with ζ mRNA 3'UTR truncations. Cell lysates from MA5.8 and its mutants (NEG, 740, 871, 950, 1070, 1136, 1330, 1457, WT, and AS3'UTR) were electrophoresed on 15% SDS-polyacrylamide gels using a reducing method and blotted onto a PVDF membrane. The membranes were then incubated with (a) a mouse anti-human ζ mAb (TIA-2) or (b) a hamster anti-mouse β -actin mAb followed by a peroxidase-conjugated anti-mouse IgG. After treatment with chemiluminescence-enhancing reagents, the membranes were visualized on ECL X-ray films, and the densities of the 18-kDa ζ protein bands were quantified as index values.

UCCUGCUGUAAAAUUGGCCUUCUGUUGUCAC-3') (Fig. 3 on line), we defined these MA5.8 mutants as Δ CS1, and Δ CS2 mutants, respectively.

ζ protein expression in MA5.8 mutants with ζ mRNA 3'UTR CS deletions

The expression of the ζ protein in MA5.8 mutants with ζ mRNA 3'UTR CS deletions was analyzed using a Western blot and TIA-2. As shown in Fig. 2, ζ production in both the Δ CS1 and Δ CS2 mutants was slightly higher than that in the AS3'UTR but was significantly lower than that in the WT mutant. From these observations, we concluded

that the ζ mRNA 3'UTR regions from +871 to +950 including CS1 and from +1070 to +1136 including CS2 were important for the production of ζ protein.

Expression of ζ and TCR/CD3 complex on the cell surfaces of MA5.8 mutants with ζ mRNA 3'UTR CS deletions

We investigated the expression of ζ and the TCR/CD3 complex on the cell surfaces of these MA5.8 mutants carrying ζ mRNA 3'UTR CS deletions using a FACS analysis. As shown in Fig. 3, the expression of ζ protein on the cell surfaces of both the Δ CS1 (mean channel fluorescence: 73.44) and the Δ CS2 (51.89) was much lower than that of

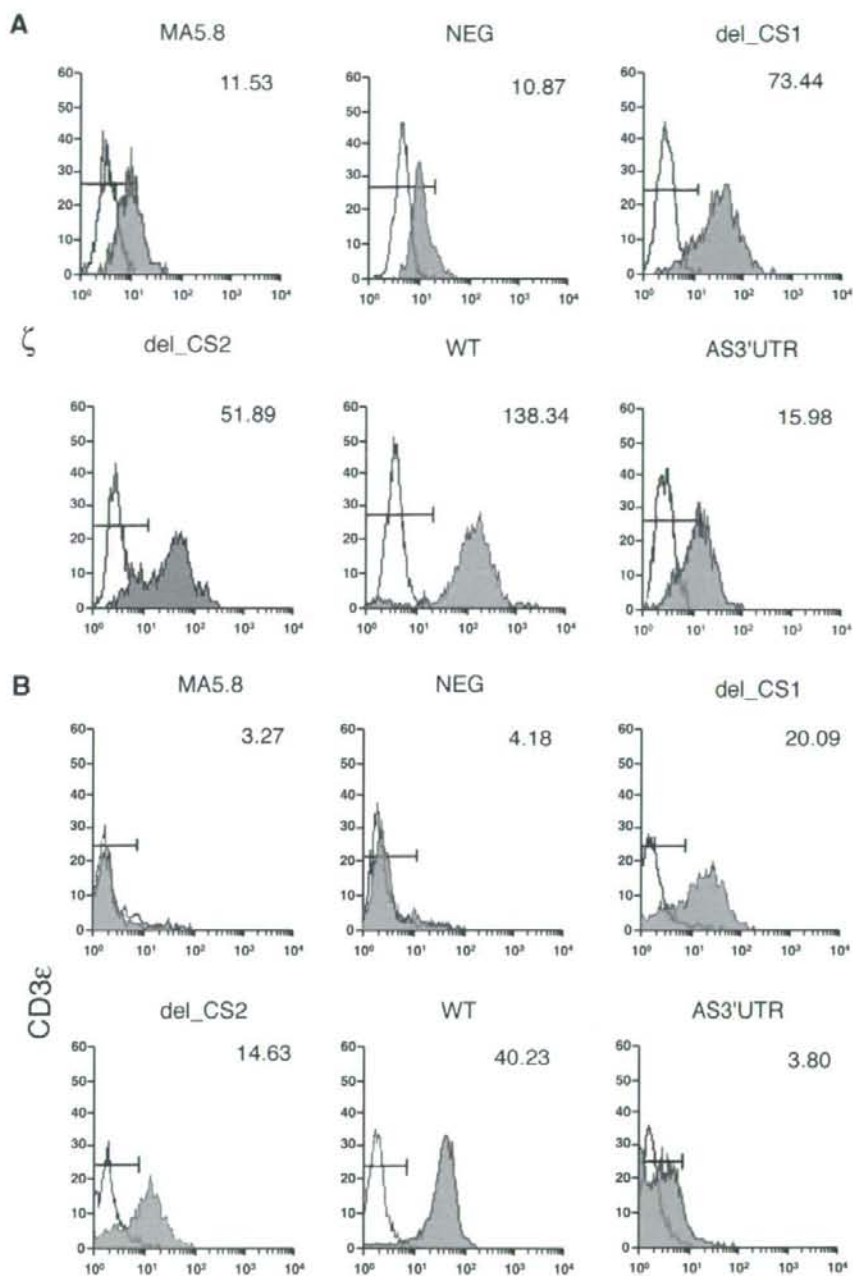


Fig. 3. Flow cytometric analysis of the MA5.8 mutants with ζ mRNA 3'UTR CS deletions. The surface expressions of (A) the ζ protein and (B) the TCR/CD3 complex on MA5.8 cells and its mutants [NEG, del_CS1(Δ CS1), del_CS2(Δ CS2), WT, AS3'UTR] were quantified using FITC-conjugated anti-human ζ mAb (TIA-2) (black profiles) and FITC-conjugated anti-mouse CD3 ϵ mAb (145-2C11) (black profiles), respectively. An FITC-conjugated mouse anti-human IgG (open profiles in (A)) or FITC-conjugated Armenian hamster anti-mouse IgG (open profiles in (B)) was used as the negative control. The mean channel fluorescence value is indicated within the figures at the *top right*.

wild-type ζ mRNA in the WT mutants. On the other hand, the stability of the CD3 ϵ mRNA was similar in the MA5.8 mutants with ζ mRNA 3'UTR CS deletions and the WT mutant.

Discussion

In this study, the down-regulation of the ζ protein in the MA5.8 mutants with ζ mRNA 3'UTR CS deletions (Δ CS1,

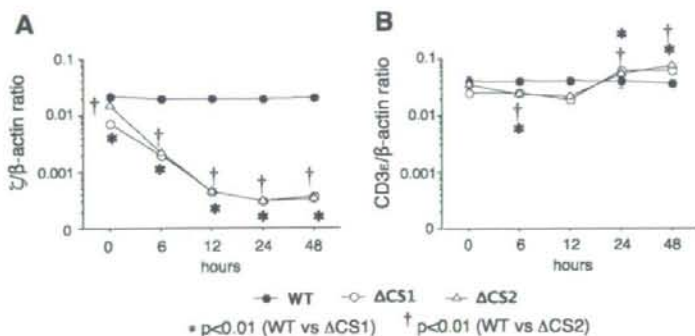


Fig. 4. Decrease in ζ mRNA stability in MA5.8 mutants with ζ mRNA 3'UTR CS deletions. MA5.8 mutants (WT, Δ CS1, Δ CS2) were cultured and incubated with 4 μ g/mL of actinomycin D in the culture media. The samples were collected at various time points, and the mRNA was subsequently extracted and converted to whole cDNA. (A) ζ and (B) CD3 ϵ cDNA were quantified using real-time PCR and were evaluated as the ratio against β -actin cDNA. Each experiment was performed in triplicate. The bars show the mean plus or minus the SD. * $p < 0.01$ for Δ CS1 (open circles) versus WT (closed circles). † $p < 0.01$ for Δ CS2 (open triangles) versus WT (closed circles).

Δ CS2) as confirmed using Western blot and FACS analyses, suggested that the production of the ζ protein was low in the absence of these two CSs of the ζ mRNA 3'UTR. The expression of the TCR/CD3 complex was also decreased in MA5.8 mutants without these regions, as shown by a FACS analysis.

We and other groups have previously reported that the ζ mRNA/as-3'UTR, which is predominantly expressed in SLE T cells, is less stable than the ζ mRNA/w-3'UTR and may be responsible for the reduced expression of the TCR/CD3 complex including ζ protein in SLE T cells [17,18]. Therefore, we examined the stability of ζ mRNA to investigate the reduction in ζ protein expression in the MA5.8 mutants expressing ζ mRNA with 3'UTR CS deletions. From our observations, ζ mRNA with 3'UTR CS deletions appeared to be less stable and more easily degraded than ζ mRNA/w-3'UTR. Conceivably, the reduction in the stability of ζ mRNA with 3'UTR CS deletions may lead to a reduction in the expression of the intracellular ζ homodimer, leading to the absence of the expression of the TCR/CD3 complex on the cell surface. Also, the reduction in IL-2 production in these MA5.8 mutants revealed that the signal from the TCR was not transduced into the cytoplasm by anti-CD3 ϵ antibody stimulation in these mutants. AREs (AUUUA motifs) bind to *trans*-acting proteins and participate in either the stabilization or destabilization of the transcripts. Two AREs, which have been reported to be responsible for the stability of ζ mRNA [24], are located at positions +735 and +803 of ζ mRNA. In our study, however, both MA5.8 mutants 740 and 871 with ζ mRNA 3'UTR truncations did not express ζ protein and MA5.8 mutants Δ CS1 and Δ CS2, which contained the AREs, were easily degraded. From these observations, there might remain the possibility that the AREs at +735 and +803 are not related to the production of ζ protein or the stability of ζ mRNA. However, this possibility can be demonstrated only by

using a deletion mutant lacking AREs but containing CSs. This study is now under way in our laboratory. In conclusion, the present study suggests that the regions between +872 and +951 and between +1076 and +1106 in the ζ mRNA 3'UTR are critical for ζ mRNA stability. Gramolini et al. reported that a 171-bp region in the 3'UTR of utrophin mRNA regulates utrophin mRNA stability, since the half-life of utrophin mRNA without this region is much shorter than that of the wild-type mRNA [25]. Also, Akgül et al. reported a 59-nucleotide (nt) pentobarbital-responsive element in the 3'UTR of *Drosophila* glutathione-S-transferase D21 (gstD21) mRNA that regulates the stability of gstD21 mRNA [26].

Interestingly, CS1 and CS2 are conserved in several mammals (Fig. 3 on line). Xie et al. reported that 106 CSs in mRNA 3'UTR are involved in post-transcriptional regulation and that half of these CSs are related to AREs, while the other half of the CSs that do not contain AREs are associated with microRNAs [27]. Since both the CS1 and CS2 in the ζ mRNA 3'UTR do not include AREs (AUUUA motifs), some miRNAs might influence the stability of the ζ mRNA by binding to the CS1 and CS2 in ζ mRNA 3'UTR regions that do not contain AREs. This possibility is now being studied in our laboratory.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.12.145.

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Availability of Cardio-Ankle Vascular Index (CAVI) as a Screening Tool for Atherosclerosis

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Background A novel index, the cardio-ankle vascular index (CAVI), which reflects the stiffness of the aorta, femoral artery, and tibial artery, was recently developed by measuring brachial–ankle pulse wave velocity and blood pressure.

Methods and Results In the present study 1,014 Japanese adults from the general population were screened to clarify the correlation between CAVI and other existing markers related to atherosclerosis, including carotid intima–media thickness (CIMT) and homocysteine (HCY). CAVI was strongly associated with age in both men and women. After adjustment for age and sex, CAVI was correlated with systolic and diastolic blood pressures. In addition, CAVI was significantly correlated with total cholesterol hemoglobin A_{1c} and total HCY, as well as CIMT.

Conclusion CAVI is an appropriate screening tool for atherosclerosis, but further studies are needed to establish a convenient and effective screening system using it. (*Circ J* 2008; 72: 304–308)

Key Words: Atherosclerosis; Cardio-ankle vascular index (CAVI); Carotid intima–media thickness (CIMT); Homocysteine (HCY)

Appropriate assessment and prevention of cardiovascular disease (CVD) is 1 of the most important medical tasks worldwide. The World Health Organization has projected that the worldwide annual number of CVD Disability-adjusted life years (DALYs; a measure that combines years of potential life lost due to premature death with years of productive life lost due to disability) will reach 153 million by 2010, 169 million by 2020, and 187 million by 2030, and that CVD death will reach 18.1 million by 2010, 20.5 million by 2020, and 24.2 million by 2030! Because atherosclerosis is a major contributor to CVD, accounting for much of the mortality and morbidity, the establishment of effective and accurate strategies for screening is very important.

Recent technological advances in medical equipment have allowed noninvasive assessment of atherosclerosis in its early stages. High-resolution B-mode ultrasonography provides a noninvasive method of quantifying arterial wall thickening, and it has been shown that the carotid intima–media thickness (CIMT) is a strong predictor of CVD.¹ In addition, pulse wave velocity (PWV) has been developed as a noninvasive clinical index of aortic stiffness² and report-

edly predicts cardiovascular events and all-cause mortality in hypertensive patients and in the general population.^{3,7} However, measurement of classic PWV is technically difficult and has low reproducibility, and the data obtained vary significantly between institutions and operators because of the technical difficulty.⁸ In order to overcome these problems, the brachial-ankle PWV (baPWV), which is simple to measure and has high reproducibility, was developed and has been shown to predict the presence of coronary disease⁹ as well as correlating with CIMT.¹⁰ However, there are problems, because this method is influenced by changes in blood pressure (BP) during the examination and by the autonomic nervous system.^{8,11}

A novel index, the cardio-ankle vascular index (CAVI), which reflects the stiffness of the aorta, femoral artery, and tibial artery and involves measurement of baPWV and BP, was recently developed.^{8,12} CAVI is essentially independent of changes in BP during examination, but shows a strong correlation with systolic BP (SBP).¹² Although there are several reports of the evaluation of CAVI in patients on hemodialysis,¹² those with hyperglycemia¹³ and those who had undergone heart transplantation,¹⁴ there are few on the evaluation of CAVI as a screening tool for atherosclerosis in the general population,¹ and none about the correlation between CAVI and CIMT. Although we recently evaluated the availability of CAVI as a screening tool for atherosclerosis in young Japanese adult (mean age 21.4 years, range 18–31 years), no correlation was observed with other existing markers, including CIMT.¹⁵

Because we hypothesized that CAVI might be a useful screening tool for atherosclerosis in the general adult population, in the present study we screened a representative sample to evaluate the correlation between CAVI and other existing markers related to atherosclerosis, including CIMT and homocysteine (HCY), which is considered to be an inde-

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Table 1 Characteristics of the Study Participants

	Men (n=242)	Women (n=772)	All participants (n=1,014)
Age (years)	64.4±1.0	61.9±10.7	62.5±10.8
BMI (kg/m ²)	23.4±3.0	22.8±3.2	23.0±3.1
WC (cm)	84.9±8.8	80.5±10.1*	81.5±10.0
SBP (mmHg)	142±20	141±21	141±21
DBP (mmHg)	87±11	84±11	85±11
TC (g/L)	2.0±0.3	2.2±0.3	2.1±0.4
TG (g/L)	1.4±0.8	1.2±0.6**	1.3±0.7
HDL-C (g/L)	0.54±0.15	0.62±0.15	0.60±0.15
LDL-C (g/L)	1.1±0.3	1.3±0.3	1.3±0.3
HbA _{1c} (%)	5.1±0.8	5.0±0.6**	5.0±0.6
Creatinine (mg/L)	10.9±2.3	8.4±1.6**	9.0±2.1
UA (g/L)	64±15	49±12**	53±15
tHcy (μmol/L)	10.3±4.7	8.3±3.8**	8.7±4.1
CIMT (mm)	0.8±0.2	0.7±0.2**	0.7±0.2
CAVI	8.5±1.4	8.0±1.3	8.1±1.3

Values are mean ± standard deviation. **p*<0.05 and ***p*<0.01 vs men. BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; HbA_{1c}, hemoglobin A_{1c}; UA, uric acid; tHcy, total homocysteine; CIMT, carotid intima media-thickness; CAVI, cardio-ankle vascular index.

pendent risk factor for atherosclerosis!⁶

Methods

Subjects

Prior to this study, ethical approval was obtained from the Special Committee of Nagasaki University (project registration number 0501120073). The study was conducted during a medical screening program for members of the general population aged over 40 years, living in Goto city (total population was 44,874 in 2007), Nagasaki Prefecture, Japan. The data were collected by the staff of Nagasaki University, in cooperation with that of Goto city. After obtaining informed consent, we enrolled 1,139 Japanese adults (285 men, 854 women): 38 participants with an apparent past or present history of cerebral infarction or hemorrhage or ischemic heart disease, and 71 participants with insufficient data were excluded from the study, as were 16 participants who showed severe hypertriglyceremia (>4.0 g/L). Finally, 1,014 participants were included for further analysis (242 men, 772 women).

Data Collection and Laboratory Measurements

Height and weight were measured, and body mass index (BMI: kg/m²) was calculated as an index of obesity. SBP and diastolic BP (DBP) were recorded at rest, simultaneously with the measurement of CAVI.

Blood samples were collected from each participant after overnight fast. Serum and plasma were separated and stored at -20°C and -80°C, respectively, until assay. Serum concentrations of total cholesterol (TC), triglyceride (TG), and high-density lipoprotein-cholesterol (HDL-C) were measured by standard laboratory procedures, and low-density lipoprotein-cholesterol (LDL-C) was calculated by the Friedwald equation. In addition to fasting blood sugar and hemoglobin A_{1c} (HbA_{1c}), serum creatinine and uremic acid (UA) were measured by standard laboratory procedures. Plasma total Hcy (tHcy) levels were measured using a high-performance liquid chromatographic method developed by Garcia and Apitz-Castro!⁷

Table 2 Simple Correlation Analysis of CAVI and Other Variables

	Men	Women	All participants
BMI	-0.021	0.003	0.01
WC	0.062	0.16**	0.16**
SBP	0.21**	0.31**	0.28**
DBP	0.14*	0.17**	0.18**
TC	0.039	0.10**	0.042
TG	-0.036	0.14**	0.11**
HDL-C	0.031	-0.070	-0.080*
LDL-C	0.039	0.108**	0.050
HbA _{1c}	0.22**	0.18**	0.20**
Creatinine	0.12	0.14**	0.20**
UA	0.020	0.088*	0.13**
tHcy	0.11	0.11**	0.14**
CIMT	0.32**	0.37**	0.37**

p*<0.05 and *p*<0.01.

Abbreviations as in Table 1.

Measurement of CAVI and CIMT

CAVI was recorded using a VaseraVS-1000 vascular screening system (Fukuda Denshi, Tokyo, Japan) with the participant resting in a supine position. The principles underlying CAVI have been described by Yambe et al.⁸ ECG electrodes are placed on both wrists, a microphone for detecting heart sounds is placed on the sternum, and cuffs are wrapped around both the arms and ankles. After automatic measurements, obtained data were analyzed using VSS-10 software (Fukuda Denshi), and the values of right and left CAVI were calculated. Averages of the right and left CAVI were used for analysis.

Measurement of CIMT by ultrasonography of the left and right carotid arteries was performed by 2 medical doctors (N.T. and M.N.), using a LOGIC Book XP with a 10-MHz linear array transducer (GE Medical Systems, Milwaukee, WI, USA). A detailed protocol has been described elsewhere!⁸ Averages of left and right CIMT were calculated and used in the analysis. Intra-observer variation of CIMT (N.T., n=32) was 0.91 (*p*<0.01), and interobserver variation (N.T. vs M.N., n=41) was 0.78 (*p*<0.01).

Statistical Analysis

Results are expressed as mean ± standard deviation. Differences between women and men in the laboratory values were evaluated using the *t*-test. Multiple linear regression analysis was performed to evaluate CAVI and other existing parameters adjusted for confounding factors (age, sex, waist circumference (WC), SBP, TG, HDL-C, HbA_{1c}, creatinine, UA, tHcy and CIMT, Table 1), which showed significant correlations with CAVI by simple linear regression analysis in all subjects. Although DBP also showed a significant correlation with CAVI, it was not analyzed as a confounding factor, because of intercorrelation with SBP (*r*=0.80, *p*<0.01). Because TG and tHcy levels had a skewed distribution, logarithmic transformation was performed for the simple correlation analysis and multiple linear regression analysis. Probability values less than 0.05 were considered indicative of statistical significance. All statistical analyses were performed using SPSS v11.0 software (SPSS Japan, Tokyo, Japan).

Results

Characteristics of the study participants are shown in Table 1. The average age of the men was significantly older

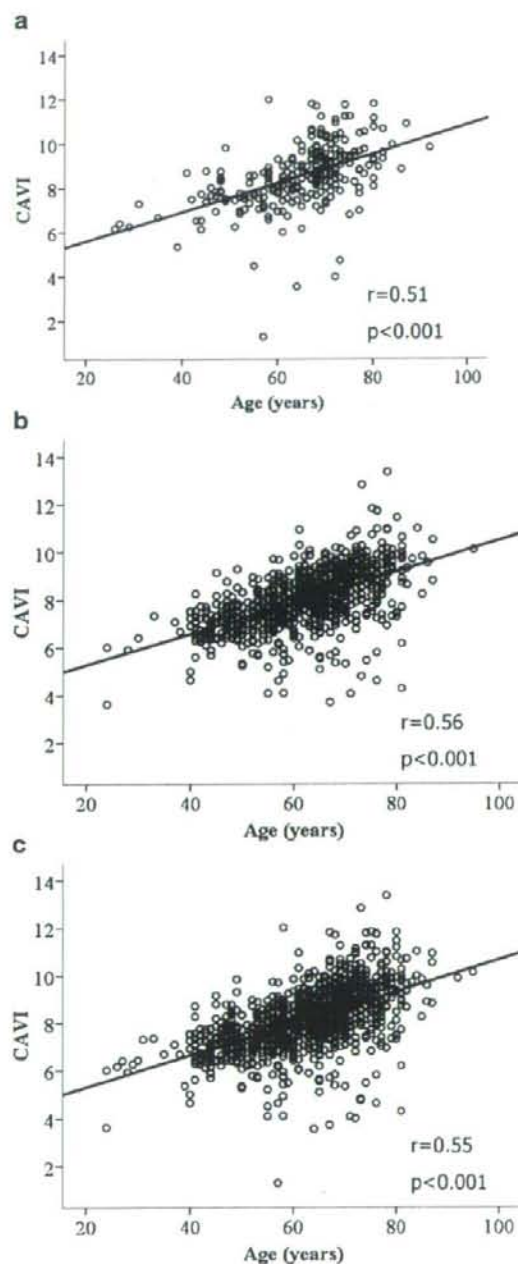


Fig 1. Relationship between cardio-ankle vascular index (CAVI) and age in (a) men ($n=242$), (b) women ($n=772$), and (c) all participants ($n=1,014$).

than that of the women (64.2 ± 11.1 years vs 61.9 ± 10.7 years, $p < 0.01$). Other than SBP and HbA_{1c}, all parameters showed significant differences between men and women.

By simple linear regression analysis, CAVI was significantly correlated with age in men ($r=0.51$, $p < 0.001$), women ($r=0.56$, $p < 0.001$), and all participants ($r=0.55$, $p < 0.001$).

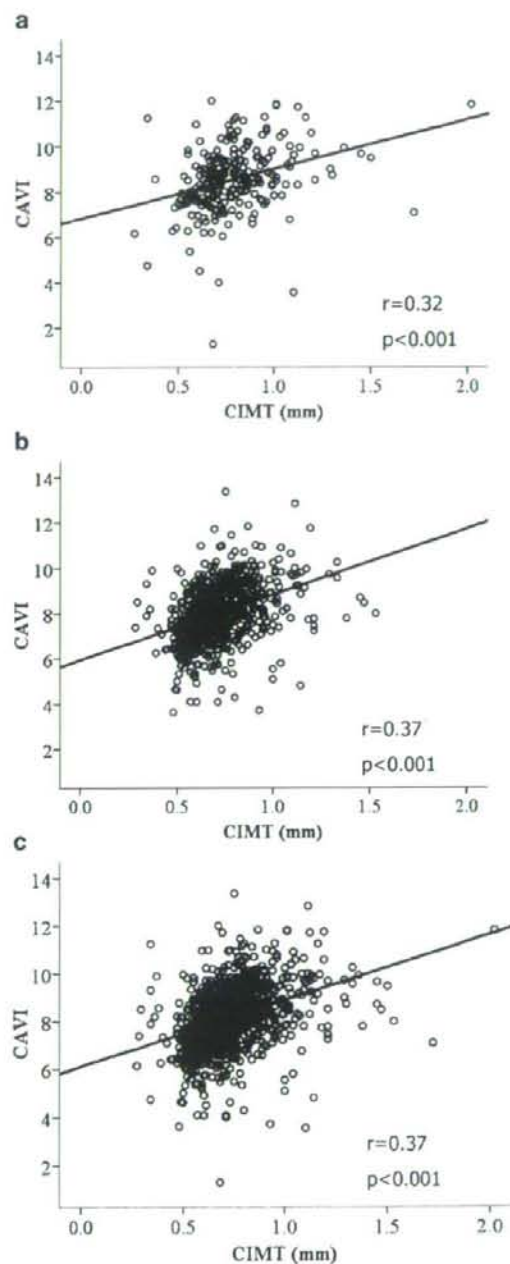


Fig 2. Relationship between cardio-ankle vascular index (CAVI) and carotid intima-media thickness (CIMT) in (a) men, (b) women, and (c) all participants.

Fig 1). It was also significantly correlated with CIMT in men ($r=0.32$, $p < 0.001$) and in women ($r=0.37$, $p < 0.001$) (Fig 2). Furthermore, CAVI was significantly correlated with SBP, DBP, HbA_{1c} and CIMT in men and with WC, SBP, DBP, TC, TG, LDL-C, HbA_{1c}, creatinine, UA, tHcy, and CIMT in women. In all participants, CAVI was signifi-

cantly correlated with WC, SBA, DBP, TG, HDL-C, HbA_{1c}, creatinine, UA, tHCY, and CIMT (Table 2).

By multiple linear regression analysis adjusted for confounding factors, CAVI was significantly correlated with age, SBP, DBP, HbA_{1c}, tHCY in all subjects (Table 3). Also, CAVI was significantly correlated with CIMT. On the other hand, CAVI was not significantly correlated with TC, TG, HDL-C, and LDL-C.

Discussion

In this study, we screened CAVI in adults from the Japanese general population and showed that it is independently associated with age, sex, SBP, HbA_{1c}, tHCY and CIMT. Previous research has shown that in vivo aortic stiffness decreases sharply with age in the first decade of life, reaching a minimum at 10 years of age, and thereafter increases with age in both genders.¹⁹ The increase in central artery stiffness with age is responsible for earlier wave reflections and changes in pressure wave contours, and cross-sectional studies have shown that aortic PWV (aPWV) increases with age by approximately $0.1 \text{ m} \cdot \text{s}^{-1} \cdot \text{year}^{-1}$.²⁰ However, we demonstrated that CAVI increases with age about 0.066/year in this study, which suggests that CAVI, as with PWV, can serve as a marker for the increase in central artery stiffness with age.

CAVI is designed not to be affected by BP during measurement, but is probably affected by long-term hypertension. By simple linear regression analysis, Shirai et al recently reported that compared with baPWV, CAVI correlated weakly with SBP and did not correlate with DBP in patients on hemodialysis.¹² However, we observed a significant correlation between CAVI and SBP/DBP in both men and women by simple linear regression analysis, and showed that CAVI independently correlated with SBP, after adjustment for confounding factors. In the general population, CAVI may reflect BP, whereas it is essentially independent of changes in BP during examination.

Although there is no doubt that LDL-C directly participates in atherosclerosis and is a major risk factor for CVD, we showed that CAVI was not significantly correlated with HDL-C or LDL-C. Previous reports also indicated a lack of association between baPWV and LDL-C,^{21,22} suggesting that further evaluation of LDL-C, in combination with apolipoprotein B, which is a reasonable index of the number of LDL particles, might be appropriate to clarify the effect of lipid metabolism on CAVI.

We are the first to show that CAVI correlates with CIMT, after adjustment for confounding factors, in the general population. Currently, measurement of CIMT is widely used as a noninvasive method of quantifying arterial wall thickening and atherosclerosis progression, and increased CIMT has been shown to be a strong predictor of cardiovascular morbidity and mortality.²³ Kobayashi et al reported that in patients with risk factors for atherosclerosis, CIMT was independently related to baPWV, and they suggested that the combination of CIMT and baPWV might give reliable information on clinical or subclinical atherosclerosis.¹⁰ Furthermore, Okura et al recently showed a significant positive correlation between CAVI and IMT by simple linear regression analysis in patients with hypertension.²⁴ As with baPWV, the measurement of CAVI requires only a few minutes for the entire procedure. Our current results suggest that measurement of CAVI in combination with CIMT might be appropriate for screening of the general

Table 3 Multiple Linear Regression Analysis of CAVI With Relevant Factors Adjusted for Confounding Factors

	β	95%CI	<i>p</i> value
Age	0.056	0.048, 0.063	<0.001
Sex	0.39	0.20, 0.59	<0.001
WC	-0.005	-0.012, 0.003	0.24
SBP	0.006	0.002, 0.009	0.002
TG	0.17	-0.20, 0.54	0.36
HDL-C	0.003	-0.002, 0.008	0.21
HbA _{1c}	0.18	0.062, 0.29	0.003
Creatinine	-0.27	-0.70, 0.15	0.21
UA	-0.012	-0.069, 0.044	0.67
tHCY	0.4	0, 0.80	0.05
CIMT	0.81	0.37, 1.26	<0.001

β , regression coefficient.

CI, confidence interval. Other abbreviations as in Table 1.

population, as well as for patients with atherosclerosis risk factors and for large-scale studies.

Interestingly, we observed that CAVI significantly correlated with HbA_{1c} in the general population after adjustment for confounding factors. It has been reported that baPWV is associated with fasting insulin levels and insulin-related factors, including HbA_{1c}, leading to reduced insulin sensitivity,²⁵ and our results suggest that, as with baPWV, CAVI may reflect glucose intolerance and possible insulin sensitivity. Further evaluation to clarify the relationship between CAVI and insulin sensitivity is needed.

After adjustment for confounding factors, CAVI was significantly correlated with tHCY, although we did not observe any differences in CAVI between the CC and CT vs TT genotypes of C667T/MTHFR. The association between baPWV and HCY is still controversial. Mayer et al evaluated the association between aPWV and tHCY in the general population and found a positive association, even after adjustment for conventional cardiovascular risk factors.²⁶ On the other hand, de Bree et al conducted a similar cross-sectional analysis and concluded that tHCY concentration was not associated with arterial stiffness.²⁷ In our study group, CIMT was significantly correlated with tHCY after adjustment for age and sex, which suggests that tHCY might be a marker relevant to atherosclerosis. However, we observed a complete lack of association of CAVI and C667T/MTHFR (data not shown), which suggests that careful elaboration is needed for the evaluation of HCY as a screening marker for atherosclerosis.

Study Limitations

First, we could not evaluate the current medication of participants, which might influence the values of CAVI and other markers, nor did we evaluate information about past/present history of coronary risk factors (hypertension, diabetes, hyperlipidemia and smoking). We also could not evaluate the relationship between CAVI and other atherosclerotic markers, such as high-sensitivity C-reactive protein and adipocytokines, including adiponectin and leptin.

Conclusion

We screened CAVI in adults from the Japanese general population, and our results indicate that it may be appropriate as a screening tool for atherosclerosis. Further studies are needed to establish a convenient and effective screening system for atherosclerosis using this technique.

Acknowledgments

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Arthritis and pneumonitis produced by the same T cell clones from mice with spontaneous autoimmune arthritis

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Keywords: animal model, interstitial lung disease, rheumatoid arthritis, T cell clone

Abstract

SKG mice, a newly established model of rheumatoid arthritis (RA), spontaneously develop autoimmune arthritis accompanying extra-articular manifestations, such as interstitial pneumonitis. To examine possible roles of T cells for mediating this systemic autoimmunity, we generated T cell clones from arthritic joints of SKG mice. Two distinct CD8⁺ clones were established and both showed *in vitro* autoreactivity by killing syngeneic synovial cells and a variety of MHC-matched cell lines. Transfer of each clone to histocompatible athymic nude mice elicited joint swelling and histologically evident synovitis accompanying the destruction of adjacent cartilage and bone. Notably, the transfer also produced diffuse severe interstitial pneumonitis. Clone-specific TCR gene messages in the inflamed joints and lungs of the recipients gradually diminished, becoming hardly detectable in 6–11 months; yet, arthritis and pneumonitis continued to progress. Thus, the same CD8⁺ T cell clones from arthritic lesions of SKG mice can elicit both synovitis and pneumonitis, which chronically progress and apparently become less T cell dependent in a later phase. The results provide clues to our understanding of how self-reactive T cells cause both articular and extra-articular lesions in RA as a systemic autoimmune disease.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology that primarily affects the synovial membranes of multiple joints (1, 2). A cardinal feature of joint inflammation in RA is proliferative inflammation of the synovium, i.e. synovitis, which leads to the destruction of adjacent cartilage and bone. In addition, RA frequently accompanies extra-articular manifestations, for example the development of rheumatoid factors, rheumatic nodules, vasculitis and interstitial lung disease (ILD). Recent studies with high-resolution imaging have indeed revealed a high prevalence of ILD in

patients with RA (3–6). RA is thus a systemic disease; yet, the immunological basis of this systemic autoimmunity is poorly understood.

T cells appear to play a key role in the development of RA as suggested by the infiltration of T cells, especially CD4⁺ T cells, into the synovial tissue of RA (7–9) and the association of genetic susceptibility to RA with particular alleles of HLA-DR (10, 11). On the other hand, there is evidence in humans and animal models that stimulated synoviocytes, composed of macrophage-like and fibroblast-like synovial cells, can

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themselves mediate joint destruction in a T cell-independent manner (12, 13). A key issue in elucidating the pathogenetic mechanism of RA is, therefore, to determine how self-reactive T cells contribute to the initiation and progression of synovitis and possibly extra-articular lesions such as ILD.

The SKG strain of mice spontaneously develops T cell-mediated chronic autoimmune arthritis (14–16). The strain possesses a mutation in the gene encoding a Src homology 2 domain of the ζ -associated protein of 70 kDa (ZAP-70), a key signal transduction molecule in T cells (17, 18). Impaired signal transduction through SKG ZAP-70 results in thymic positive selection and failure in negative selection of highly self-reactive T cells that include potentially arthritogenic T cells (14). The SKG arthritis progresses chronically, starting from small joints of the digits and symmetrically progressing to larger joints, such as the wrists and ankles. Histologically, affected joints show hyperplasia of synovocytes, inflammatory cell infiltration, pannus formation and destruction of cartilage and bone, eventually leading to joint deformity. As extra-articular lesions, they develop interstitial pneumonitis, dermatitis, necrobiotic nodules akin to rheumatic nodules in RA and systemic vasculitides. Serologically, they spontaneously develop IgM-type rheumatoid factors, auto-antibodies against type II collagen and antibodies cross-reactive with *Mycobacterium tuberculosis* heat shock protein (hsp) 70. IL-1, tumor necrosis factor (TNF)- α , IL-6 or IL-17 deficiency inhibits the development of arthritis in SKG mice (15, 19), similar to the effects of anti-cytokine therapies in RA (20, 21). Thus, autoimmune disease in SKG mice closely resembles RA in clinical and immunopathological characteristics. In addition, considering recent findings that genetic polymorphism of a signaling molecule at a TCR proximal step involving ZAP-70 significantly contributes to the susceptibility to RA and other autoimmune diseases (22, 23), SKG mice can be a suitable model for elucidating how a T cell-intrinsic anomaly contributes to the development of RA as a systemic autoimmune disease.

In this study, we have attempted to determine the role of T cells in SKG autoimmune disease by establishing T cell clones from their arthritic lesions. We have established two distinct CD8⁺ clones and show that both of them have the potential to induce not only arthritis but also pneumonitis. This indicates that inflammation in both the joints and the lung can be mediated, at least in part, by common autoreactive T cell clones in SKG mice. In addition, by adoptively transferring these T cell clones to normal mice, we show that autoreactive T cells are able to initiate arthritis; yet, the arthritis can progress apparently in a T cell-independent manner in a later phase. These findings contribute to our understanding of how T cells cause chronic arthritis and ILD in RA.

Materials and methods

Mice

SKG and (SKG \times BALB/c)F₁ mice (14) were maintained in the animal facility of Kyoto University under a microbially conventional condition. Female C.B-17 SCID mice (Clea Japan, Tokyo, Japan), DBA/1J, BALB/c and BALB/c-nu/nu mice (Charles River Japan, Kanagawa, Japan) were maintained under specific pathogen-free conditions at Kyoto

University or Discovery Research Laboratories of Shionogi & Co., Ltd. All experiments were approved by the Animal Care and Use Committee at Kyoto University and Shionogi & Co., Ltd.

Culture medium

The culture medium for SKG T cell lines and clones was AIM-V supplemented with 20% RPMI-1640, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol (ME), 2 mM L-glutamine, \times 1 penicillin/streptomycin (Gibco BRL, Gaithersburg, MD, USA), 10% heat-inactivated FCS (Hyclone, Logan, UT, USA), 10% rat T-STIMTM with Con A (Becton Dickinson, Franklin Lakes, NJ, USA), 100 U/ml of recombinant mouse IL-2 (Genzyme, Cambridge, MA) and 5 μ g/ml of Con A (Sigma, St Louis, MO, USA).

Establishment of T cell clones from arthritic joints of SKG mice

To establish T cell lines, severely swollen joints of SKG mice were aseptically excised, finely minced and cultured until clusters of mononuclear cells were confirmed in bulk culture. Outgrown T cells were cloned in 96-well microplates by using SKG synovial cells (1×10^3) as feeder cells. Synovial cells were prepared as previously described (16). Briefly, synovial tissues from wrist and ankle joints were digested with 400 Mandl U/ml of Liberase Brendzyme II (Roche) in RPMI-1640 medium for 1 h at 37°C; digested cells were filtered through a nylon mesh to prepare single-cell suspensions. A typical composition of the synovial cell preparation was ~10% CD11b⁺ monocyte/macrophages, ~20% Gr-1⁺ granulocytes, ~1% T cells and other cells. Several days later non-adherent cells were removed by washing the plates with culture medium. T cells that had outgrown from the bulk culture of synovial cells were dispensed at 1, 5, 20 or 50 cells per well and apparently single colonies were propagated in the culture medium described above. Clonality of each cell was confirmed by microplate hybridization assay (MHA) (24) and sequence analysis of TCR. Established T cell clones were maintained without feeder cells. Dengue 2F7 and 3F2 T cell clones, established by immunization of BALB/c mice with the NS3 peptide of dengue virus, were kindly provided by Dr H. Masaki (Kinki University). All cultures were performed in a humidified atmosphere of 7.5% CO₂ at 37°C.

Cytokine detection

Cytokine production by T cell clones were analyzed by ELISA. T cell clones were stimulated with 10 ng/ml of phorbol myristate acetate (PMA) (Wako Chemicals USA, Inc., Richmond, VA, USA) and 0.4 μ g/ml of ionomycin (Calbiochem, Darmstadt, Germany) in culture medium at 1×10^6 cells/ml for 16 h. The supernatants were assayed for various cytokines using specific ELISA kits (Endogen, Woburn, MA, USA, and Axis-Shield, Oslo, Norway) according to the manufacturer's protocol. Cytokine mRNA levels in the joints and lungs of clone recipient mice were analyzed by quantitative PCR as described previously (25).

MHA for TCR AV and BV family and sequence analysis

MHA, cDNA synthesis and PCR amplifications of TCR of each T cell clone were performed as described previously (24). The