Original Article

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Multiplex cytokine analysis of Werner syndrome

Makoto Goto[1,2,3,*], Koichiro Hayata[2], Junji Chiba[2], Masaaki Matsuura[4,5], Sachiko Iwaki-Egawa[6], Yasuhiro Watanabe[6]

[1] *Division of Anti-ageing and Longevity Sciences, Department of Medical Technology, Faculty of Medical Engineering, Toin University of Yokohama, Yokohama, Japan;*
[2] *Department of Orthopaedics & Rheumatology, East Medical Center, Tokyo Women's Medical University, Tokyo, Japan;*
[3] *Division of Rheumatology, Nerima-Hikarigaoka Hospital, Tokyo, Japan;*
[4] *Department of Cancer Genomics, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan;*
[5] *Graduate School of Public Health, Teikyo University, Tokyo, Japan;*
[6] *Department of Life Sciences, School of Pharmacy, Hokkaido Pharmaceutical University, Hokkaido, Japan.*

Summary **We reported a minor inflammation-driven ageing (inflammageing) assessed by highly sensitive CRP (hsCRP) in normal individuals and patients with Werner syndrome (WS), followed by an ageing associated Th2-biased cytokine change in normal ageing in the previous papers. To further study the association of hsCRP and 26 cytokines/chemokines in 35 WS patients, a multiple cytokine array system was used in the same serum samples as were examined for hsCRP. The serum levels of Th2 cytokines (IL-4, IL-6, IL-10, and GM-CSF), Th1 products (IL-2, TNFα, IL-12, and IFNγ) and monocyte/macrophage products (MCP-1, basic FGF and G-CSF) in WS were significantly elevated compared with normal ageing. Elevated hsCRP level in WS was significantly correlated with IL-6, IL-12 and VEGF levels, if age and sex were taken into account. A pro-inflammatory cytokine/chemokine circuit-stimulated immunological shift to Th2 in WS was similar to normal ageing. These cytokine/chemokine changes may induce a systemic chronic inflammation monitored by hsCRP, though these immunological changes in WS were more complicated than normal ageing, possibly due to the WS-specific chronic inflammation such as skin ulcer, diabetes mellitus and central obesity with visceral fat deposition. Further study may warrant the pathophysiology of Th2 shift and Th2-biased inflammageing in normal ageing and WS.**

Keywords: Ageing, inflammageing, Werner syndrome, CRP, cytokine, chemokine

1. Introduction

Werner syndrome (WS; MIM#27770), the genetically-determined progeroid syndrome has been extensively studied as the representative natural model of human ageing. We have reported the elevation of inflammatory markers in WS in a series of publications irrespective of the apparent inflammation (*1*).

Our recent study indicated a significant ageing-associated increase in the serum level of highly sensitive CRP (hsCRP) in the normal Japanese individuals and the serum hsCRP level was also significantly elevated in WS compared with age-matched normal adult (NA) control and normal elderly (NE) population from both sexes (*2*).

Human ageing is inevitably accompanied by an increasing chance of environmental attack from inside (such as mutants, endoplasmic reticulum (ER) stress and by-products associated with immune- surveillance activity) and outside (such as ultra violet light, air pollution, allergens, infectious agents, drugs and foods), producing a minor inflammation that is widely recognized as a patho-physiologically fundamental metabolism to generate energy with thermogenesis, leading to tissue development, wound healing or tissue destruction during healthy development and ageing (*3-6*).

Ageing-associated inflammation coined as "inflammageing" has been monitored mainly by hsCRP (*7-9*). Inflammageing is probably caused by an imbalance

**Address correspondence to:*
Dr. Makoto Goto, Department of Orthopaedics & Rheumatology, East Medical center, Tokyo Women's Medical University, 2-1-10 Nishi-Ogu, Arakawa-Ku, Tokyo 116-8567, Japan.
E-mail: werner.goto@gmail.com

between an increase in pro- and a decrease in anti-inflammatory cytokines/chemokines, leading to ignite the ageing-related conditions including diabetes mellitus (DM), sarcopenia, osteoporosis, cancer, atherosclerosis, cognitive decline and finally death (*3,8,10*).

Ageing-associated changes of pro/anti-inflammatory cytokines/chemokines have been reported by using ELISA and multiplex technology. However, the results are conflicting. Ageing-associated elevation of pro-inflammatory cytokines/chemokines including interleukin (IL)-6, IL-8 (CXCL8), tumor necrosis factor α (TNFα), macrophage inhibitory protein-1α (MIP-1α: CCL3) and monocyte chemoattractant protein-1 (MCP-1: CCL2) was reported by Mariani *et al* (*11*). However, both Shurin *et al*. (*12*) and Kim *et al*. (*13*) described no ageing-associated changes of these cytokines/chemokines. Shurin *et al*. (*12*) reported a significant age-associated increase of interferonγ inducible protein-10 (IP-10: CXCL10) and eotaxin (CCL11). Elevation of IL-6, MCP-1 and IP-10 was described by Miles *et al*. (14), Inadera *et al*. (*15*) and Antonelli *et al*. (*16*).

In our preceding paper, the serum levels of IL-4, IL-6, IL-13, IL-15, granulocyte-macrophage- colony stimulating factor (GM-CSF), interferon-γ (IFNγ), IP-10 and TNFα were significantly correlated with normal ageing (manuscript submitted). In contrast, IL-2, IL-8, MIP-1α levels were negatively associated with normal ageing. The Th2 products: IL-6 and IL-13 levels were significantly associated with serum level of hsCRP in normal ageing, if age and sex were taken into account. Cytokine/chemokine analysis in WS has never been reported.

The aim of this study was to *i*) compare the serum levels of 26 cytokines/chemokines examined by multiplex assay in WS with the apparently healthy Japanese volunteers; and *ii*) clarify the association of 26 cytokines/chemokines with the increased level of hsCRP in WS.

2. Materials and Methods

2.1. *Study population*

All the samples studied in the present experiment were collected between 2000 and 2010, and were the same sera as were used in the previous hsCRP study (*2*). A total of 35 serum samples from the patients with mutation-proven WS aged between 32 and 70 years were used. All of the WS patients showed the characteristic manifestations as previously described: typical body status/face, hoarseness, gray hair/alopecia, skin hyper/hypo-pigmentation, sarcopenia, cataract, osteoporosis, and subcutaneous calcification. As some WS patients had diabetes mellitus (DM) and skin ulcers (SU), the patients were sub-grouped into 1) SU (+) DM (+) (*n* = 14), 2) SU (+) DM (-) (*n* = 12), 3) SU (-) DM (+) (*n* = 4), and 4) SU (-) DM(-) (*n* = 5) based on their phenotypes as indicated in Table 1.

Table 1. Clinical characteristics in Werner syndrome patients

Subgroups	SU*	DM**	ID	Age	Sex
1	+	+	WS12901	32	F
1	+	+	WS57201	37	M
1	+	+	WS19201	38	M
1	+	+	WS56301	39	M
1	+	+	WS57801	41	M
1	+	+	WS51301	42	M
1	+	+	WS19201	44	M
1	+	+	WS6301	46	M
1	+	+	WS53601	46	M
1	+	+	WS58501	51	M
1	+	+	WS58301	53	M
1	+	+	WS54801	57	M
1	+	+	WS56201	70	M
1	+	+	WS1801	70	M
2	+	-	WS6103	32	M
2	+	-	WS6104	32	M
2	+	-	WS14501	35	M
2	+	-	WS51601	36	F
2	+	-	WS53101	38	F
2	+	-	WS53901	43	F
2	+	-	WS53801	46	F
2	+	-	WS2101	50	F
2	+	-	WS55801	53	F
2	+	-	WS52901	54	F
2	+	-	WS54001	57	F
2	+	-	WS4701	59	F
3	-	+	WS58701	35	M
3	-	+	WS57701	38	F
3	-	+	WS57401	41	M
3	-	+	WS4401	41	M
4	-	-	WS5801	43	M
4	-	-	WS0402	47	M
4	-	-	WS7501	48	M
4	-	-	WS0401	49	F
4	-	-	WS10501	52	F

*SU: skin ulcer. **DM: diabetes mellitus. 26 had skin ulcers (SU) and 18 diabetes mellitus (DM). Subgroups: 1) SU (+) DM (+), *n* = 14; 2) SU (+) DM (-), *n* = 12; 3) SU (-) DM (+), *n* = 4; 4) SU (-) DM (-), *n* = 5.

All of the individuals provided written informed consent for this study, which was approved by the ethics committee of Toin University of Yokohama. All of the samples were stored at -80°C until use. For statistical comparison, 113 normal individuals were divided into two groups according to their age: normal adult (NA) aged between 25 and 70 years (*n* = 57; M = 29, F = 28) and normal elderly (NE) aged between 71 and 100 years (*n* = 56; M = 12, F = 44). All the normal individuals including NE were the same as examined in the previous study and met the SENIEUR criteria (*17*).

2.2.1. *Multiplex cytokine array system*

Serum levels of 26 cytokines/chemokines including IL-1β, IL-1 receptor antagonist (ILra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, basic fibroblast growth factor (basic FGF), granulocyte-colony stimulating factor (G-CSF), GM-CSF, platelet derived growth factor (PDGF), vascular endothelial

growth factor (VEGF), TNFα, IFNγ, IL-8, IP-10, MCP -1, MIP-1α, MIP-1β (CCL4) and eotaxin were simultaneously measured using commercially-available bead-based immunofluorescence Bio-Plex Suspension Array System (BioRad; Hercules, CA) according to the manufacturer's instruction.

Briefly, six distinct sets of fluorescently dyed beads loaded with capture monoclonal antibodies specific for each cytokine/chemokine to be tested, were used. Serum samples (50 µL/well of fourfold diluted serum) or standards (50 µL/well) were incubated with 50 µL of premixed bead sets into the pre-wetted 96 well microtiter plates at 4°C. After incubation and washing, 25 µL of fluorescent detection antibody mixture was added for 30 min and then the samples were washed and resupended in assay buffer.

High standard curves for each soluble factor were used. The low standard curves were obtained by tenfold diluted high standards. The formation of different sandwich immune-complexes was obtained by using the Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad; Hercules, CA). A 50 µL volume was sampled by each well and the fluorescent signal of a minimum of 100 beads per region (cytokine/chemokine) was evaluated and recorded. Values presenting a coefficient of variation beyond 10% were discarded before the final analysis.

2.2.2. *Determination of hsCRP*

The data of hsCRP used in this study was obtained in the previous experiment (*2*) by using CircuLex high-sensitivity CRP ELISA kit (MBL Woburn, MA) according to the user's manual.

2.3. *Data analysis and statistics*

Differences of serum cytokines/chemokines between WS and healthy individual groups (NA and NE) were evaluated by Wilcoxon rank sum test. We examined ageing-associated changes of serum levels of cytokines/chemokines using regression analyses expressed as

$$\log\_e(\text{cytokine/chemokine}(j)) = a + b^{*}\,\text{Age},$$

where a is an estimated intercept, b is an estimated regression coefficient for Age and j is an indicator for individual cytokine/chemokine. To examine the relationship between serum levels of hsCRP and cytokine/chemokine, we performed regression analyses expressed as

$$\log\_e(\text{hsCRP}) = a + b^{*}\log\_e(\text{cytokine/chemokine}(j)),$$

where a is an estimated intercept, b is an estimated regression coefficient and j is an indicator for individual cytokine/chemokine. Multiple regression models were used to further examine the relationship between hsCRP and cytokines/chemokines with adjustment of sex and age effects on the serum levels. The model (a) was expressed as

$$\log\_e(\text{hsCRP}) = a + b_1{*}\text{Age} + b_2{*}\text{Sex} + b_3{*}\log\_e(\text{cytokine/chemokine}(j)),$$

where a (intercept), b_1, b_2 and b_3 are estimated regression coefficients and j is an indicator for individual cytokine/chemokine. The model (b) was expressed as

$$\text{hsCRP} = \exp\{a + b1{*}\text{Age} + b2{*}\text{Sex} + b3\text{cytokine/chemokine}(j)\},$$

where a (intercept), $b1$,$b2$ and $b3$ are estimated regression coefficients and j is an indicator for individual cytokine/chemokine. We used Akaike's Information Criterion (AIC) (*18*) for model selection between models with original data and models with log-transformed values (not shown). We show only results based on models with log-transformed values described above because they were better than models with original data. Statistical language R (*19*) was used for the analyses. p-values < 0.05 were considered to be statistically significant. Differences of serum cytokine/chemokine levels between subgroups in WS were estimated by Welch's two-sample t-test with unequal variances.

Serum cytokine/chemokine data were analyzed using the Bio-Plex manager software version 5.0 (Bio-Rad; Hercules, CA). Standard levels between 70 and 130% of the expected values were considered to be accurate and were used. In general, at least six standards were accepted and used to establish standard curves following a five-parameter logistic regression model (5PL), Sample concentrations were immediately interpolated from the standard curves. Values were expressed as pg/mL and presented as mean ± S.E.

3. Results

3.1. *Cytokine/chemokine levels between Normal adult (NA) and Normal elderly (NE) groups*

As indicated in Table 2, serum levels of IL-4, IL-6, IL-13, IL-15, GM-CSF, IP-10, MCP-1 and TNFαin NE group were significantly elevated compared with NA group. In contrast, serum levels of IL-1β, IL-1ra and MIP-1αin NE group were significantly decreased compared with NA group. The rest of the cytokine/chemokine levels examined were comparable between NA and NE.

3.2. *Cytokine/chemokine levels in WS*

Single regression analyses of 26 cytokines/chemokines

Table 2. Cytokine/chemokine changes between NA, NE and WS

Cytokines/chemokines (pg/mL)	Normal adult (NA) (mean ± S.E.; *n* = 35)	WS (mean ± S.E.; *n* = 35)	Normal elderly(NE) (mean ± S.E.; *n* = 56)	*p* value		
				NA *vs.* NE	WS *vs.* NA	WS *vs.* NE
IL-4	6.4 ± 0.4	8.8 ± 0.9	8.6 ± 0.9	0.008**	0.008**	0.113
IL-6	11.6 ± 3.2	408.6 ± 381.5	22.1 ± 8.1	< 0.001***	< 0.001***	0.007**
IL-13	9.7 ± 1.0	13.9 ± 1.8	16.7 ± 2.0	0.002**	0.057	0.429
IL-15	4.4 ± 0.8	7.5 ± 1.2	9.5 ± 0.9	< 0.001***	< 0.001***	0.046*
GM-CSF	33.9 ± 9.9	117.4 ± 37.5	99.6 ± 17.4	< 0.001***	< 0.001***	0.932
IP-10 (CXCL10)	862.4 ± 51.2	1898.3 ± 530.8	1503.6 ± 175.6	< 0.001***	0.164	0.083
MCP-1 (CCL2)	46.1 ± 6.2	53.2 ± 4.1	51.5 ± 4.5	0.010**	0.008**	0.431
TNFα	32.9 ± 9.1	39.9 ± 7.8	38.1 ± 7.6	0.020*	0.002**	0.113
IL-5	2.53 ± 0.62	2.67 ± 0.31	2.82 ± 1.05	0.245	0.078	0.007**
IL-9	38.37 ± 17.08	101.07 ± 71.8	42.04 ± 9.78	0.328	0.121	0.549
IL-10	2.59 ± 0.34	3.66 ± 0.45	2.93 ± 0.31	0.303	0.031*	0.149
basicFGF	22.09 ± 1.54	31.48 ± 2.72	24.47 ± 2.45	0.982	0.002**	0.004**
G-CSF	10.80 ± 0.69	16.84 ± 1.58	14.73 ± 4.31	0.673	< 0.001***	< 0.001***
IFNγ	158.97 ± 31.48	192.9 ± 22.58	226.36 ± 45.16	0.086	0.021*	0.259
VEGF	114.68 ± 9.99	247.4 ± 51.29	165.56 ± 15.31	0.074	0.094	1
Eotaxin (CCL11)	106.31 ± 7.48	94.45 ± 12.0	117.63 ± 9.23	0.282	0.278	0.079
IL-1β	6.4 ± 4.0	10.8 ± 8.7	1.7 ± 0.4	0.041*	0.502	0.005**
IL-1ra	59.9 ± 4.6	72.4 ± 8.9	48.6 ± 5.4	0.024*	0.355	0.011*
IL-2	3.8 ± 0.7	5.07 ± 1.37	2.99 ± 0.97	0.373	0.029*	< 0.001***
IL-7	8.1 ± 0.71	10.93 ± 1.37	7.92 ± 0.44	0.469	0.128	0.157
IL-12	19.82 ± 1.45	30.42 ± 5.09	18.35 ± 1.72	0.26	0.303	0.091
IL-17	28.56 ± 2.08	31.23 ± 3.23	25.58 ± 1.49	0.25	0.213	0.071
MIP-1α (CCL3)	9.6 ± 1.9	5.6 ± 0.6	4.9 ± 0.7	< 0.001***	0.247	0.053
MIP-1β (CCL4)	209.27 ± 47.63	125.37 ± 10.93	133.77 ± 7.29	0.308	0.772	0.455
IL-8 (CXCL8)	164.51 ± 83.30	17.63 ± 1.88	34.76 ± 15.81	0.172	0.262	0.189
PDGF	18015.1 ± 2613.1	7482.1 ± 887.2	12540.5 ± 1475.7	0.877	0.082	0.007**

Cytokine/chemokine levels between NA, NE and WS were tested by Wilcoxon rank sum test: *:$p < 0.05$, **:$p < 0.01$, ***:$p < 0.001$.

showed no significant age-associated changes in WS, as was already inferred in the previous hsCRP paper (*2*).

In WS patients, serum levels of IL-2, IL-6, basic FGF, and G-CSF were significantly elevated compared with NA and NE (Table 2). Levels of IL-4, IL-15, GM-CSF, MCP-1, TNFα, IL-10 and IFNγin WS were significantly increased compared with NA. Both IL-1β and IL-1ra levels were significantly elevated in WS in comparison with NE. Serum levels of PDGF, IL-5 and IL-15 in WS were significantly decreased compared with NE. The rest of the cytokine/chemokine levels (IL-7, IL-8, IL-9, IL-12, IL-17, eotaxin and MIP-1β) were comparable between NA, NE and WS.

3.3. *Association of cytokine/chemokine with serum hsCRP in WS*

Using multiple regression models, temporal effect of age on the serum level of hsCRP was determined. The Table 3-a and 3-b showed estimated regression coefficients with S.E., and *p*-values.

IL-6 was significantly associated with hsCRP. The relationship between IL-6, hsCRP and ageing according to the model (a) was log_e (hsCRP) = -1.199 + 0.04 × Age + 0.339 × Sex + 0.317 × log_e (IL-6) (Table 3-a).

Both IL-12 and VEGF were also significantly associated with hsCRP according to the model (b); hsCRP = exp{1.311 + 0.01 × Age + 0.522 × Sex + 0.007 × IL-12} and hsCRP = exp{1.26+ 0.01 × Age + 0.64 × Sex + 0.001 × VEGF} (Table 3-b), respectively. In these formulae, Sex was 1 for male and 0 in female. No sex difference was observed concerning to the ageing associated changes of 26 cytokines/chemokines examined.

3.4. *Association of cytokines/chemokines with clinical phenotypes in WS*

In the WS patients, the serum hsCRP level was similar between SU (+) and SU (-) groups or DM (+) and DM (-) groups, and among SU (+) DM (+), SU (+) DM (-) , SU (-) DM (+) and SU (-) DM(-) subgroups, as was reported in the previous paper (*2*).

Significant differences were in eotaxin (111.8 ± 14.0 *vs*. 44.4 ± 13.2 pg/mL, $p < 0.05$), IP-10 (2348.6 ± 693.2 *vs*. 597.4 ± 183.2 pg/mL, $p < 0.05$) and MIP-1α (6.2 ± 0.8 *vs*. 3.9 ± 0.7 ng/mL, $p < 0.05$) between SU (+) and SU (-), respectively. Serum levels of eotaxin (121.0 ± 17.6 *vs*. 66.3 ± 13.4 pg/mL, $p < 0.05$) and G-CSF (20.4 ± 2.1 *vs*. 13.1 ± 2.1 pg/mL, $p < 0.01$) were significantly elevated in DM (+) group compared with DM (-) group, respectively.

Among subgroups, most cytokine/chemokine levels including IL-1ra, IL-5, IL-6, eotaxin, G-CSF and IP-10 in group1 were significantly elevated compared with group 4 (Table 4). The serum levels of IL-1β, IL-1ra,

Table 3-a. Association of cytokine/chemokine with hsCRP in WS

Dependent variable	Independent variables	Estimated regression coefficient	S.E.	*p* value
Log (hsCRP)	Intercept	-1.199	1.062	0.268
	Age	0.040	0.019	0.048*
	Sex	0.339	0.389	0.386
	IL-6	0.317	0.132	0.022*

Model (a): log_e(hsCRP) = $a + b1^*$Age + $b2$+Sex + $b3^*$log_e(Cytokine(j)), a was an intercept, $b1^*$, $b2^*$ and $b3^*$ were estimated regression coefficients, and j was an indicator for individual cytokine. Sex was 1 for male and 0 for female. Significance level. *: $p < 0.05$.

Table 3-b. Association of cytokine/chemokine with hsCRP in WS

Dependent variable	Independent variables	Estimated regression coefficient	S.E.	*p* value
hsCRP	Intercept	1.311	0.766	0.097
	Age	0.01	0.014	0.463
	Sex	0.522	0.350	0.146
	IL-12	0.007	0.003	0.029*
hsCRP	Intercept	1.260	0.770	0.112
	Age	0.010	0.014	0.472
	Sex	0.640	0.357	0.082
	VEGF	0.001	0.000	0.018*

Model (b): hsCRP = exp{ $a + b1^*$Age + $b2^*$Sex + $b3^*$Cytokine(j) }, a was an intercept. $b1^*$, $b2^*$, and $b3^*$ were estimated regression coefficients, and j was an indicator for individual cytokine. Sex was 1 for male and 0 for female. Significance level. *: $p < 0.05$

Multiple regression analyses expressed as log_e (hsCRP) = $a + b1^*$Age + $b2^*$Sex + $b3^*$ log_e (cytokine (j)) (model (a)) and hsCRP = exp{$a + b1^*$Age + $b2^*$Sex + $b3^*$ cytokine (j) } (model (b)) were indicated, where a is an estimated intercept, $b1^*$, $b2^*$, and $b3^*$ were estimated regression coefficients and j is an indicator for individual cytokine/chemokine. In these formulae, Sex was 1 for male and 0 in female.

The relationship between IL-6, hsCRP and ageing according to the model(a) was log_e (hsCRP) = -1.199 + 0.04 × Age + 0.339 × Sex + 0.317 × log_e (IL-6) (Table 3-a).

Both IL-12 and VEGF were also significantly associated with hsCRP according to the model (b); hsCRP = exp{ 1.311 + 0.01 × Age + 0.522 × Sex + 0.007 × IL-12} and hsCRP = exp{ 1.26+ 0.01 × Age + 0.64 × Sex + 0.001 × VEGF}, respectively.

IL-5 and IL-13 in group 2 were significantly elevated compared with group 4. Interestingly, MCP-1 level in group1 was significantly decreased compared with group 4. The rest of the cytokines/chemokines levels including IL-2, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10, IL-12, IL-15, IL-17, basic FGF, IFNγ, MIP-1α, MIP-1β, TNFα and VEGF were comparable between subgroups.

4. Discussion

The serum levels of Th1 products (TNFα), Th2 products (IL-4, IL-6, and GM-CSF) and monocyte/macrophage products (IL-15 and MCP-1) were elevated with normal ageing and more elevated in WS. In addition, IL-13 and IP-10 were increased with normal ageing. Ageing-associated elevations of serum IL-13 and serum IL-15 have never been reported in normal human ageing. Although the pro-inflammatory monocyte/macrophage products including IL-1β, IL-1ra and MIP-1αwere decreased with normal ageing, some monocyte/macrophage products (IL-1β, IL-1ra, basic FGF and G-CSF), and Th1 products such as IL-2 and IFNγ, and Th2 cytokine IL-10 were increased in WS.

The WS patients with more inflammatory phenotypes (SU (+) and DM (+)) produced more pro-inflammatory cytokines/chemokines such as IL-1β, IL-1ra, IL-5, IL-6, IL-13, eotaxin, IP-10 and G-CSF than less inflammatory subgroups in WS. Immunological shift to Th2-type T cells was common between normal ageing and WS, although Th1-type cytokines, monocyte/macrophage origin chemokines were also elevated in WS.

One of the characteristic phenotypes in WS is the central obesity with visceral fat deposition (*1*) irrespective of DM and the incidence of central obesity in normal Japanese has increased recently with ageing (*20*).

The WS with inflammatory phenotypes such as SU, DM and central obesity may induce more complicated immunological changes than normal ageing, though both have a common immunological shift to Th2.

CRP is the prototypical acute-phase reactant in man and has been proposed as a marker of atherosclerosis-associated diseases including coronary heart disease and cerebro-vascular accidents (*21-23*). As CRP has an antagonistic pleiotropic activity, CRP induced by IL-6 can act as pro-inflammatory by producing TNF-αand IL-1β (*24*). CRP can also function as a protective machinery by activating the classical pathway of complement system (*25*), enhancing phagocytosis (*26*) and binding to the Fcγreceptors on leukocytes, leading to the anti-inflammatory cytokine IL-10 production and the suppression of IL-12 secretion (*27,28*) as a component of the innate immune system.

Among these cytokines/chemokines, the serum levels of Th2 products (IL-6 and IL-13), IL-15 and

Table 4. Serum cytokines/chemokines in Werner syndrome from different sugroups

Cytokine/chemokine	Subgroups	Mean (pg/mL)	S.E.	*p* value matrix		
				Group 2	Group 3	Group 4
IL-1β	Group 1: SU(+)DM(+) (*n* = 14)	23.1	21.7	0.643	0.645	0.095
	Group 2: SU(+)DM(-) (*n* = 12)	3.8	1.9	-	0.446	0.048*
	Group 3: SU(-)DM(+) (*n* = 4)	1.1	0.3	0.446	-	0.413
	Group 4: SU(-)DM(-) (*n* = 5)	0.8	0.3	0.048*	0.413	-
IL-1ra	Group 1: SU(+)DM(+) (*n* = 14)	87.9	14.6	0.504	0.327	0.008**
	Group 2: SU(+)DM(-) (*n* = 12)	75.1	13.8	-	0.671	0.04*
	Group 3: SU(-)DM(+) (*n* = 4)	66.6	33.6	0.671	-	0.389
	Group 4: SU(-)DM(-) (*n* = 5)	27	10.1	0.04*	0.389	-
IL-5	Group 1: SU(+)DM(+) (*n* = 14)	3.1	0.5	0.742	0.327	0.007**
	Group 2: SU(+)DM(-) (*n* = 12)	2.9	0.5	-	0.684	0.035*
	Group 3: SU(-)DM(+) (*n* = 4)	2.6	1.2	0.684	-	0.286
	Group 4: SU(-)DM(-) (*n* = 5)	1	0.4	0.035*	0.286	-
IL-6	Group 1: SU(+)DM(+) (*n* = 14)	985.7	953.4	0.251	0.798	0.021*
	Group 2: SU(+)DM(-) (*n* = 12)	28	12	-	0.316	0.442
	Group 3: SU(-)DM(+) (*n* = 4)	29	9.2	0.316	-	0.063
	Group 4: SU(-)DM(-) (*n* = 5)	9.8	3	0.442	0.063	-
IL-13	Group 1: SU(+)DM(+) (*n* = 14)	13.6	2.4	0.56	0.878	0.156
	Group 2: SU(+)DM(-) (*n* = 12)	15.7	2.4	-	0.684	0.014*
	Group 3: SU(-)DM(+) (*n* = 4)	18.7	10.9	0.684	-	0.556
	Group 4: SU(-)DM(-) (*n* = 5)	6.7	3.3	0.014*	0.556	-
Eotaxin	Group 1: SU(+)DM(+) (*n* = 14)	137.9	19.7	0.036*	0.035*	0.002**
	Group 2: SU(+)DM(-) (*n* = 12)	61.3	16.5	-	0.77	0.091
	Group 3: SU(-)DM(+) (*n* = 4)	61.9	23.2	0.77	-	0.286
	Group 4: SU(-)DM(-) (*n* = 5)	30.3	13.8	0.091	0.286	-
G-CSF	Group 1: SU(+)DM(+) (*n* = 14)	19.5	2	0.071	0.721	0.014*
	Group 2: SU(+)DM(-) (*n* = 12)	14.9	2.5	-	0.202	0.225
	Group 3: SU(-)DM(+) (*n* = 4)	23.3	6.9	0.202	-	0.063
	Group 4: SU(-)DM(-) (*n* = 5)	8.9	3	0.225	0.063	-
IP-10	Group 1: SU(+)DM(+) (*n* = 14)	3431.6	1220.5	0.322	0.158	0.044*
	Group 2: SU(+)DM(-) (*n* = 12)	1085	206.5	-	0.379	0.195
	Group 3: SU(-)DM(+) (*n* = 4)	783.4	328.8	0.379	-	0.73
	Group 4: SU(-)DM(-) (*n* = 5)	448.6	208.7	0.195	0.73	-
MCP-1	Group 1: SU(+)DM(+) (*n* = 14)	49.1	5.4	0.899	0.878	0.034*
	Group 2: SU(+)DM(-) (*n* = 12)	50.3	7.9	-	1	0.13
	Group 3: SU(-)DM(+) (*n* = 4)	48.6	9.2	1	-	0.19
	Group 4: SU(-)DM(-) (*n* = 5)	75.6	10.4	0.13	0.19	-

Cytokine/chemokine levels among subgroups were estimated by two-sample *t*-test with unequal variances. Significance level: $^{*}p < 0.05$, $^{**}p < 0.01$.

IP-10 were significantly associated with serum level of hsCRP, if age and sex were taken into account in normal ageing (manuscript submitted). However, serum hsCRP was significantly associated with IL-6, IL-12 and VEGF in WS, as indicated in the present study.

IL-4, IL-6 and IL-10 are pro-inflammatory cytokines produced by Th2-type T cells, B cells, classically activated macrophages, adipose- tissue-associated macrophages, fibroblasts and endothelial cells, possibly leading to the activation of wound healing macrophages for tissue repair with fibrosis (*29*) and the abrogation of autophagy and autophagy-mediated killing of intracellular mycobacteria in human macrophages (*5,30*).

Pro-inflammatory chemokine: MCP-1 has been reported to be the products from adipose-tissue-associated macrophages, classically activated macrophages, fibroblasts, endothelial cells and mast cells (*29,31*). Although an increase in the serum levels of IP-10 and MCP-1 with normal ageing has already been described by others (*14,16*), an elevation of serum IL-13 and IL-15 has never been described. IL-13 is aTh2-derived mediator of allergic inflammation and IL-15 is a monocyte/macrophage product from viral infection to proliferate natural killer cells of innate immunity.

These cytokine/chemokine distributions may suggest an association of monocyte/macrophage products-stimulated Th2 type inflammation leading to tissue remodeling and fibrosis by wound healing macrophages in WS and also with normal ageing as suggested by others (*11,29,31,32*).

The elevating inflammation associated with normal ageing may not be the direct result of one-way traffic destruction of tissues, but the sum result of ongoing tissue degradation and repair by a cytokine/chemokine circuit-driven inflammation and regeneration (*33*).

Immunological shift to Th2-type T cells with normal

ageing and WS may stimulate a pro-inflammatory cytokine/chemokine circuit, leading to a systemic chronic inflammation monitored by hsCRP. Monocyte/macrophage products including MCP-1 can be an immunologically possible candidate to stimulate Th2-type T cell shift, though we did not observe a significant association between hsCRP and MCP-1 if age and sex were taken into account.

Further study may be needed to clarify the pathogenesis of Th2 shift and Th2-biased mild inflammation: inflammageing in normal ageing and WS.

In conclusion, minor inflammation-driven inflammageing in WS monitered by hsCRP is associated with increases in IL-6, IL-12 and VEGF.

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References

1. Goto M, Miller RW. From premature gray hair to helicase-Werner syndrome: Implications for aging and cancer. In: Monograph on Cancer Research. No.49, Japan Scientific Societies press & Karger, Tokyo, Japan, 2001.
2. Goto M, Sugimoto K, Hayashi S, Ogino T, Sugimoto M, Furuichi Y, Matsuura M, Ishikawa Y, Iwaki-Egawa S, Watanabe Y. Aging-associated inflammation in healthy Japanese individuals and patients with Werner syndrome. Exp Gerontol. 2012; 47:936-939.
3. Charo IF, Taubman MB. Chemokines in the pathogenesis of vascular diseases. Circ Res. 2004; 95:858-866.
4. Kollmann TR, Levy O, Montgomery RR, Goriely S. Innate immune function by Toll-like receptors: Distinct responses in newborns and the elderly. Immunity. 2012; 37:771-783.
5. Kolattukudy PE, Niu J. Inflammation, endoplasmic reticulum stress, autophagy, and the monocyte chemoattractant protein-1/CCR2 pathway. Circ Res. 2012; 110:174-189.
6. Lee P, Werner CD, Kebebew E, Celi FS. Functional thermogenic beige adipogenesis is inducible in human neck fat. Int J Obes (Lond). 2014; 38:170-176.
7. Franceschi C, Bonafe M, Valensin S, Olivieri F, De Luca M, Ottaviani E, De Benedictis G. Inflamm-aging. An evolutionary perspective on immunosenescence. Ann NY Acad Sci. 2000; 908:244-254.
8. Goto M. Inflammaging (inflammation + aging): A driving force for human aging based on an evolutionarily antagonistic pleiotropy theory? BioSci Trends. 2008; 2:218-230.
9. Balkwill F, Coussens LM. Cancer: An inflammatory link. Nature. 2004; 431:405-406.
10. Shaw AC, Goldstein DR, Montgomery RR. Age-dependent dysregulation of innate immunity. Nat Rev Immunol. 2013; 13:875-887.
11. Mold C, Rodriguez W, Rodic-Polic B, Du Clos TW. C-reactive protein mediates protection from lipopolysaccharide through interactions with Fc gamma R. J Immunol. 2002; 169:7019-7025.
12. Shurin GV, Yurkovetsky ZR, Chatta GS, Tourkova IL, Shurin MR, Lokshin AE. Dynamic alteration of soluble serum biomarkers in healthy aging. Cytokine. 2007; 39:123-129.
13. Kim HO, Kim HS, Youn JC, Shin EC, Park S. Serum cytokine profiles in healthy young and elderly population assessed using multiplexed bead-based immunoassays. J Transl Med. 2011; 9:113.
14. Miles EA, Rees D, Baneriee T, Cazzola R, Lewis S, Wood R, Oates R, Tallant A, Cestaro B, Yagoob P, Wahle KW, Calder PC. Age-related increase in circulating inflammatory markers in men are independent of BMI, blood pressure and blood lipid concentrations. Atherosclerosis. 2008; 196:298-305.
15. Inadera H, Egashira K, Takemoto M, Ouchi Y, Matsushima K. Increase in circulating levels of monocyte chemoattractant protein-1 with aging. J Inteferon Cytokine Res. 1999; 19:1179-1182.
16. Antonelli A, Rotondi M, Fallahi P, Ferrari SM, Paolicchi A, Romaqnani P, Serio M, Ferrannini E. Increase of CXC chemokine CXCL10 and CC chemokine CCL2 serum levels in normal ageing. Cytokine. 2006; 34:32-38.
17. Ligthart GJ, Corberand JX, Fournier C, Galanaud P, Hijmans W, Kennes N, Muller-Hermelink HK, Steinmann GG. Admission criteria for immunogerontological studies in man: The SENIEUR protocol. Mech Ageing Dev. 1984; 28:47-55.
18. Akaike H. Information theory and extension of the maximum likelihood principle. B.N. Petrov, F. Csaki (Eds.), Proceedings of the 2nd International Symposium on Information Theory, Akademiai Kiado, Budapest, 1973; pp. 267-281.
19. Ihaka R, Gentleman R. R: A language for data analysis and graphics. J Comp Grap Stat. 1996; 5:299-314.
20. Sakurai T, Iimuro S, Araki A, Umegaki H, Ohashi Y, Yokono K, Lto H. Age-associated increase in abdominal obesity and insulin resistance, and usefulness of AHA/NHLBI definition of metabolic syndrome for predicting cardiovascular disease in Japanese elderly with type 2 diabetes mellitus. Gerontology. 2010; 56:141-149.
21. Pai JK, Pischon T, Ma J, Manson JE, Hankinson SE, Joshipura K, Curhan GC, Rifai N, Cannuscio CC, Stampfer MJ, Rimm EB. Inflammatory markers and the risk of coronary heart disease in men and women. N Engl J Med. 2004; 351:2599-2610.
22. Wakugawa Y, Kiyohara Y, Tanizaki Y, Kubo M, Ninomiya T, Hata J, Doi Y, Okubo K, Oishi Y, Shikata K, Yonemoto K,Maebuchi D, Ibayashi S, Iida M. C-reactive protein and risk of first-ever ischemic and hemorrhagic stroke in a general Japanese population: The Hisayama study. Stroke. 2006; 37:27-32.
23. Ishikawa J, Tamura Y, Hoshide S, Eguchi K, Ishikawa S, Shimada K, Kario K. Low-grade inflammation is a risk factor for clinical stroke events in addition to silentcerebral infarcts in Japanese older hypertensives: The Jichi Medical School ABPM Study, wave 1. Stroke. 2007; 38:911-917.
24. Kishimoto T. IL-6: From its discovery to clinical

applications. Int Immunol. 2010; 22:347-352.
25. Kaplan MH, Volanakis JE. Interaction of C-reactive protein complexes with the complement system. I. Consumption of human complement associated with the reaction of C-reactive proteinwith pneumococcal C-polysaccharide and with the choline phosphatides, lecithin andsphingomyelin. J Immunol. 1974; 112:2135-2147.
26. Bharadwaj D, Stein MP, Volzer M, Mold C, Du Clos TW. The major receptor for C-reactive protein on leukocytes is fcgamma receptor II. J Exp Med. 1999; 190:585-590.
27. Kim S, Elkon KB, Ma X. Transcriptional suppression of interleukin-12 gene expression following phagocytosis of apoptotic cells. Immunity. 2004; 21:643-653.
28. Du Clos TW. C-reactive protein as a regulator of autoimmunity and inflammation, Arthritis Rheum. 2003; 48:1475-1477.
29. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol. 2008; 8:958-969.
30. Harris J, De Haro SA, Master SS, Keane J, Roberts EA, Delgado M, Deretic V. T helper 2 cytokines inhibit autophagic control of intracellular Mycobacterium tuberculosis. Immunity. 2007; 27:505-517.
31. Mansfield AS, Nevala WK, Dronca RS, Leontovich AA, Shuster L, Markovic SN. Normal ageing is associated with an increase in Th2 cells, MCP-1(CCL1) and RANTES (CCL5), with differences in sCD40L and PDGF-AA between sexes. Clin Exp Immunol. 2012; 170:186-193.
32. Loke P, Gallagher I, Nair MG, Zang X, Brombacher F, Mohrs M, Allison JP, Allen JE. Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection. J Immunol. 2007; 179:3926-3936.
33. Goto M. Emerging concept of ageing: Inflammageing. Inflammation and Regeneration. 2009; 29:249-257.

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