Highly sensitive ELISA for soluble Fas in serum: increased soluble Fas in the elderly

MITSURU SEISHIMA,* MASAO TAKEMURA, KUNIKAISAITO, HIROTAKESANO,1 SHINYAMINATOGUCHI,1 HISAYOSHI FUJIWARA,1 TAKAHISA HACHIYA,2 and AKINO NOMB

We have developed and characterized a highly sensitive ELISA for soluble Fas (sFas) in the serum. The linearity of calibrator range was 0.06–2.00 μg/L and the detection limit was 0.01 μg/L. The average within- and between-run CVs were 3.9% and 3.8%, respectively. The recovery of added sFas to serum was 93–118%. The effects of possible interferences (tryglyceride, hemoglobin, bilirubin) were negligible. We determined serum sFas in 155 healthy subjects, ages 20–69. The mean value of sFas in men (2.50 ± 0.63 μg/L, n = 78) was significantly higher than that in women (2.01 ± 0.53 μg/L, n = 77) (P < 0.001). Furthermore, there was a significant correlation between serum sFas concentration and age (men, r = 0.397, P < 0.001; women, r = 0.569, P < 0.001). Although the concentrations of sFas tended to increase with aging, it remains to be clarified how Fas-mediated apoptosis relates to aging.

INDEX TERMS: apoptosis • aging • transmembrane protein • tumor necrosis factor receptor

Apoptosis is clearly characterized by controlled autodigestion of the cell, unlike necrosis, which is a pathologic form of cell death resulting from acute cellular injury. Recent studies show that the failure of cells to undergo apoptosis is involved in the pathogenesis of various diseases, including cancer, autoimmune disorders, and viral infections [1]. Accelerated apoptosis, on the contrary, might be involved in AIDS and neurodegenerative disorders [2]. Fas antigen, a member of the tumor necrosis factor (TNF) receptor superfamily, is a transmembrane protein expressed in a variety of tissues including liver, heart, lung, kidney, and thymus [3]. Fas has been shown to trigger apoptosis in susceptible target cells when bound to its physiological ligand [4] or to agonistic anti-Fas antibodies [5, 6]. More recently, a secreted form of Fas (soluble Fas; sFas) has been identified [7]. In general, this and other soluble forms of receptors are produced either through the proteolytic cleavage of membrane-bound receptors, as is the case for human necrosis factor receptor [8], or as translation products of alternatively spliced mRNA, as is the case for human interleukin-7 receptor [9]. These secreted receptors can bind with the corresponding ligand in a way similar to that of their membrane-bound counterparts. The resulting decrease in Fas-mediated apoptosis may contribute to the pathogenesis of the above-mentioned disorders.

So far to our knowledge, only Mountz et al. have reported serum sFas concentrations [7, 10]. They showed [7] that sFas concentrations in patients with systemic lupus erythematosus or rheumatoid arthritis are significantly higher than those in 10 healthy individuals. Afterward, they corrected their estimation of sFas in 32 control subjects [10]. However, no detailed information is available on the population of control subjects in this report. In the present study, we have developed and characterized a highly sensitive ELISA for serum sFas and determined sFas in 155 healthy subjects, ages 20–69.

Materials and Methods

BLOOD SAMPLES

Blood samples were taken from healthy subjects (78 men and 77 women), ages 20–69. All sera were separated by low-speed centrifugation (800g, 15 min) at 4 °C and stored at -70 °C until analysis. Procedures followed were in accordance with the ethical guidelines of our institute.

SOLUBLE FAS ELISA

Polystyrene plates (Maxisorp; Nunc, Roskilde, Denmark) were precoated with rabbit IgG for anti-human Fas synthetic peptide (amino acid no. 305–319). This polyclonal rabbit anti-Fas antibody was generated by repeated injection of 200 μg of Fas peptide combined with Freund's complete adjuvant, and the specificity of this antibody was confirmed by Western blotting.
(described later). Each well of a polystyrene plate was blocked with 10 g/L bovine serum albumin in phosphate-buffered saline (10 mmol/L phosphate, 150 mmol/L saline, pH 7.4) (BSA-PBS). Sera were diluted 1:5 in PBS containing 100 mL/L normal rabbit serum, and 100 mL of the diluted sample was applied to each well. The plate was incubated with mild shaking (200 rpm) at room temperature for 1 h. After incubation, the plate was washed five times with PBS containing 0.5 mL/L Tween 20, and 100 mL of horseradish peroxidase-conjugated anti-human Fas mouse monoclonal antibody developed by Yonehara et al. [11] was added and incubated with shaking at room temperature for 1 h. The plate was then washed five times with PBS containing 0.5 mL/L Tween 20, and incubated for 30 min with 100 mL of freshly prepared tetramethylbenzidine (1.0 mmol/L) in 10 mmol/L citric acid buffer, pH 5.5, containing hydrogen peroxide (3.5 mmol/L per well). The reaction was terminated by addition of 100 mL of 6 mol/L phosphate. The absorbance was read at 450 nm with a microplate reader (SLT-210; Labinstruments, Glodig, Austria). The results were calculated from the best-fitting least-squares parabola with an on-line microcomputer (NEC 9801-V2M; NEC Co., Tokyo, Japan). All samples were assayed in duplicate.

STATISTICAL ANALYSIS
The data are expressed as mean ± SD, unless otherwise indicated, and the statistical analysis was performed by unpaired Student’s t-test or ANOVA. Correlation coefficients were obtained by linear regression analysis.

Results

CALIBRATION FOR SFAS
The sFas was prepared from WR19L-12a (mouse T-cell line transfected with human sFas cDNA) [5] by using affinity chromatography conjugated with mouse anti-Fas monoclonal antibody generated by Yonehara et al. [11]. The column was washed with Tris-buffered saline [50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 10 mL/L Nonidet P-40, 5 g/L sodium deoxycholate, 1 g/L sodium dodecyl sulfate (SDS)] and the sFas bound to the column was eluted by 200 mL/L glycine-HCl buffer (pH 2.3) containing 100 mL/L glycerol. The purity of sFas was confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) with 125 g/L acrylamide. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (CBBR) and destained with 10% acetic acid. The sFas showed a specific band with an apparent Mr of 46 000 (Fig. 1), and the protein bands on the gel were scanned by a densitometer (Model GS-670; Bio-Rad Labs., Hercules, CA). The concentration of purified sFas was determined by comparison with the protein concentration of BSA (Fig. 1).

SPECIFICITY OF ANTIBODY TO SFAS
The specificity of antibodies used in this study was confirmed by Western blotting. The sFas from WR19L-12a cells was detected as a single band corresponding to Mr, 46 000 by either mouse monoclonal or rabbit polyclonal antibodies. No band was detected in the WR19L cell extract, which does not express sFas (Fig. 2).

Fig. 1. SDS-PAGE pattern of sFas and BSA at various concentrations.

The gel was stained with 0.5% CBBR-250 and destained with 10% acetic acid. The sFas and BSA were electrophoresed at the positions of Mr, 46 000 and 66 000, respectively. Lane 1, calibration markers; lanes 2–5, 1, 0.5, 0.25, and 0.125 μg of BSA, respectively; lanes 6–9, 20, 10, 5, and 2.5 μL of purified sFas respectively. The sFas concentration was 40 mg/L with BSA as a protein calibrator.

CALIBRATION CURVE AND DETECTION LIMIT
Soluble Fas was diluted with PBS containing 100 mL/L normal rabbit serum to concentrations ranging from 0.06 to 2.00 μg/mL (Fig. 3). The detection limit of this assay was 0.01 μg/L, which is the concentration corresponding to a signal 3 SD above the mean for a blank calibrator.

LINEARITY
Linearity was studied by analyzing serial dilutions of two individual samples. Dilutions up to 64-fold were assayed (Fig. 4). Up to 32-fold dilution, measured values ranged from 100% to 115% of expected values.

PRECISION
We tested the precision of the assay by measuring five sera 20 times in one assay (within-run) and 20 consecutive assays

Fig. 2. Western blotting of sFas after SDS-PAGE.

The sFas from WR19L-12a was detected as a single band of Mr, 46 000. Antigen: Lanes 1 and 3, WR19L-12a cell extract; lanes 2 and 4, WR19L cell extract. Antibody: Lanes 1 and 2, anti-sFas mouse monoclonal antibody; lanes 3, and 4, anti-Fas peptide rabbit IgG.
Table 1. Precision of sFas assay.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean ± SD, µg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>0.77 ± 0.06</td>
<td>6.49</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1.70 ± 0.05</td>
<td>2.35</td>
</tr>
<tr>
<td>Sample 3</td>
<td>4.47 ± 0.11</td>
<td>2.46</td>
</tr>
<tr>
<td>Sample 4</td>
<td>6.67 ± 0.27</td>
<td>3.89</td>
</tr>
<tr>
<td>Sample 5</td>
<td>8.45 ± 0.37</td>
<td>4.38</td>
</tr>
<tr>
<td>Between-run</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 6</td>
<td>0.96 ± 0.06</td>
<td>6.25</td>
</tr>
<tr>
<td>Sample 7</td>
<td>1.86 ± 0.06</td>
<td>3.23</td>
</tr>
<tr>
<td>Sample 8</td>
<td>2.33 ± 0.10</td>
<td>4.29</td>
</tr>
<tr>
<td>Sample 9</td>
<td>5.42 ± 0.15</td>
<td>2.77</td>
</tr>
<tr>
<td>Sample 10</td>
<td>7.34 ± 0.18</td>
<td>2.45</td>
</tr>
</tbody>
</table>

SERUM SFAS IN MEN AND WOMEN
The sFas concentrations in men (2.50 ± 0.63 µg/L, n = 78) were significantly higher than those in women (2.01 ± 0.53 µg/L, n = 77) (P < 0.001). When compared in each age group, sFas concentrations in men significantly exceeded those in women in all age groups except 60–69, and sFas concentrations in this group were significantly higher than those of the 20–29 age group both in men (2.27 ± 0.37 vs 2.95 ± 0.68 µg/L, P < 0.01) and women (1.64 ± 0.30 vs 2.56 ± 0.45 µg/L, P < 0.001). Furthermore, sFas concentrations were significantly correlated with age (r = 0.397, P < 0.001 for men and r = 0.569, P < 0.001 for women) (Fig. 6).

Fig. 3. Calibration curve for sFas.

Fig. 4. Assay linearity for sFas on stepwise dilution of two serum samples.

Fig. 5. Effects of triglyceride (A), hemoglobin (B), and conjugated (C) and unconjugated (D) bilirubin on sFas assay were studied in three samples with different sFas concentrations.

(between-run). The results are shown in Table 1. The average within- and between-run CVs were 3.9% and 3.8%, respectively.

ANALYTICAL RECOVERY
In experiments on analytical recovery of sFas, we used four individual sera (1.55, 3.99, 4.93, and 6.17 µg/L sFas). We assayed each sample in duplicate after addition of three different amounts of sFas (0.51, 1.11, and 3.87 µg/L). Calculated recovery range was 93% to 118% of added sFas.

INTERFERENCE STUDIES
We tested the effects of possible interferences on this assay with a kit (Interference Check-A; Kokusa-Shiyaku Co., Kobe, Japan). The effects of triglyceride (turbidity), hemoglobin, and conjugated and unconjugated bilirubin on the assay were examined in three serum samples with different concentrations (Fig. 5). The effects of these interferences were negligible.


Seishima et al.: Soluble Fas determination by ELISA

Discussion

We developed a highly sensitive ELISA system for serum sFas determination. Precision, recovery, and linearity were all excellent. In addition, the assay was not disturbed by possible interfering substances, and the results were all accurate. Our sFas values of 2.26 ± 0.63 μg/L in 155 healthy subjects were much lower than those in a report by Cheng et al. (~30 μg/L in 10 healthy individuals) [7]. However, they corrected their previous estimation of normal concentrations of sFas to 1.3 ± 0.3 μg/L [11]. Although the reason for this discrepancy is unclear, it may be due to differences of the calibrator or specificity of the antibody to sFas. Furthermore, their study is still small-scale (n = 32 for control subjects), and the difference of the population studied may also be related.

Apoptosis is involved in a variety of biological processes, including embryo maintenance of tissue homeostasis [7]. The Fas antigen was cloned by Itoh et al. [5] as a cell-surface antigen shown to trigger apoptosis in susceptible target cells when bound to its physiological ligand [4] or to agonistic anti-Fas antibodies [6]. This secreted form of Fas may competitively inhibit Fas–Fas ligand interactions on the cell, whereas the exact mechanism by which sFas is released from the cell surface is unknown. The resulting decrease in Fas-mediated apoptosis may contribute to the pathogenesis of autoimmune diseases [7].

Of interest in the present study are the age- and sex-related changes in serum sFas concentrations, although its physiological significance remains obscure. Zhou et al. [12] demonstrated that Fas expression decreases with age in mouse thymocytes and spleen cells. They also observed that Fas function itself decreases more than Fas expression on lymphocytes. A possible reason is that alternatively spliced or nonfunctional forms of the Fas might be present in aged mice, because such a Fas could prevent Fas-mediated apoptosis [3]. Accordingly, the present results of high sFas in the elderly may support their hypothesis. This suppressed intrathymic Fas signaling with age leads to increases in thymocyte apoptosis by a Fas-independent pathway, and defective Fas signaling may play a role in development of thymic atrophy with aging, as suggested by Zhou et al. [12]. However, the exact mechanism of sFas release from cells has not been elucidated. It may be secreted directly in a spliced form [9] or may be cleaved from the cell membrane by a metalloproteinase such as TNF receptor [8]. In any case, Fas release might be accelerated in the elderly in spite of low Fas expression, although its biological and clinical significance is not clear at present. It will also be necessary to take account of the interaction between sFas and Fas ligand in a further study. Indeed, the sFas ligand derived from the T-cell lineage has also been defined [13].

In conclusion, this ELISA system is highly sensitive, and sFas determination by this assay will help clarify the involvement of Fas-mediated apoptosis in various disorders. Additionally, age- and sex-related changes in serum sFas should be examined for the evaluation of sFas in various disorders.

References