Available online at www.annclinlabsci.org

Microplate ELISAs for Soluble VCAM-1 and ICAM-1

Pi-Yueh Chang,[1] Tsu-Lan Wu,[1] Kuo-Chien Tsao,[1] Chia-Chi Li,[1] Chien-Feng Sun,[1] and James T. Wu[2]
[1] Department of Pathology, Chang Gung Memorial Hospital, Taipei, Taiwan
[2] ARUP Laboratories, Department of Pathology, University of Utah Medical Center, Salt Lake City, Utah

Abstract. Soluble vascular cell adhesion molecule (sVCAM-1) and soluble intercellular adhesion molecule (sICAM-1) are adhesion molecules that are detectable in the serum of patients with cancer, cardiovascular diseases (CVD), and type 2 diabetes. This report describes enzyme-linked immunosorbent assays (ELISAs) on microplates for sVCAM-1 and sICAM-1. The ELISAs have the sandwich test format; polyclonal antibodies are coated on microwells and a one-step procedure is used in which the serum specimen and detecting antibody are added simultaneously to an antibody-coated well. These assays both use HRP-conjugated sheep anti-mouse-IgG to generate the color for quantification. Sensitivities for detecting sVCAM-1 and sICAM-1 are 49 and 40 ng/ml, respectively. Coefficients of variation for within-day and day-to-day replicate analyses are <10%. Results by these in-house ELISAs for serum sVCAM-1 and sICAM-1 compared well with those obtained with commercial kits from R&D Systems, Inc. (correlation coefficients = 0.98 and 0.99 for sVCAM-1 and sICAM-1, respectively). Reference values for serum sVCAM-1 and sICAM-1 levels were measured in 369 apparently healthy Chinese adults, age 30 to 79 yr. There was no significant effect of gender on the reference values for sVCAM-1 or sICAM-1. Serum sVCAM-1 levels (mean ± SD) were higher in subjects 60 yr old (625 ± 126 ng/ml), compar ed to those <60 yr old (525 ± 110 ng/ml) (p <0.001). Age did not significantly affect the reference values for serum sICAM-1 levels (mean ± SD, 249 ± 86 ng/ml). The authors believe that these simple, inexpensive ELISAs will be useful for assessing the risks for development of cancer, CVD, and type 2 diabetes. *(received 3 February 2005; accepted 8 February 2005)*

Keywords: sVCAM-1, sICAM-1, adhesion molecules, ELISA, microtiter plate

Introduction

Inflammation, the body's natural defense against microbial invasion or injury, is recognized as a risk factor for the development of many diseases [1]. This is especially true of chronic inflammation, which has recently been found to play a role in the pathogenesis of cancers, cardiovascular diseases (CVD), type 2 diabetes, and many other diseases [2-4].

At the site of inflammation, expression of various adhesion molecules can usually be detected. The expression of adhesion molecules is upregulated by proinflammatory cytokines through signal transduction pathways, such as activation of a transcription factor, NF-κB [5]. Among the various adhesion molecules, vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1, CD 54) are products of the immunoglobin gene superfamily involved in adhesion on endothelial and epithelial cells. Increased expression of VCAM-1 and ICAM-1 is associated with endothelial dysfunction in patients with CVD [21]. Elevated concentrations of soluble VCAM-1 and ICAM-1 have been reported in patients with type 2 diabetes and those with cancer [2,6]. Moreover,

Address correspondence to James T. Wu, Ph.D., ARUP Laboratories, 500 Chipeta Way, Salt Lake City, UT 84108, USA; tel 801 583 2787; fax 801 584 5207; e-mail wuj@aruplab.com.

Abbreviations: BSA. bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRP, horse radish peroxidase; sICAM, soluble intercellular adhesion molecule; NF-κB, nuclear transcription factor-kappa B; PBS, phosphate buffered saline; TMB, tetramethylbenzidine (K-Blue); sVCAM, soluble vascular cell adhesion molecule.

inflammatory diseases are associated with various expression patterns of VCAM-1 and ICAM-1, suggesting that they have different pathogenic roles.

Adhesive interactions are implicated in tumor progression and metastasis, enabling tumor cells to establish metastatic colonies [6]. Elevated circulating adhesion molecules in serum have been detected in Hodgkin's disease [7], and cancers of the bladder [8], lung [9], thyroid [10], urinary tract [11], liver [12], and ovary [13].

We have begun to recognize that expression of adhesion molecules on the arterial endothelium recruits leukocytes to the site of a lesion, which is an early event in inflammation and precedes plaque formation in atherosclerosis [14-17]. For example, expression of VCAM-1 occurs in response to endothelial dysfunction, recruiting the adhesion of leukocytes to injured endothelial cells; the subsequent migration of leukocytes across the endothelium is a key step in the pathway to atherogenesis. Increased circulating levels of adhesion molecules may be an early signal for atherosclerosis [18]. Adhesion molecules might conceivably represent therapeutic targets because of their key roles in the pathogenesis of various diseases.

Inflammation is a major risk factor for type 2 diabetes [19,20]. Circulating adhesion molecules such as sVCAM-1 and sICAM-1 have been proposed as early markers for the development of type 2 diabetes [21].

In this study, we have developed ELISAs on microtiter plates for measuring serum sICAM-1 and sVCAM-1 levels. Our experience suggests that it is important to measure sICAM-1 and sVCAM-1 simultaneously for risk assessment of various diseases. Measuring the pattern of sVCAM-1 and sICAM-1 responses has also been emphasized by Iiyama et al [22] in a study of experimental atherogenesis in animals [22].

In addition to developing ELISAs for sVCAM-1 and sICAM-1, we have also established reference values using serum specimens from apparently healthy Chinese subjects. We found that individuals 60 yr old hav e significant higher serum levels of sVCAM-1. No significant differences were found between men and women in the reference values for serum levels of either adhesion molecule.

Materials and Methods

Reagents. Sheep anti-human ICAM-1 polyclonal antibody (pAb) and sheep polyclonal anti-human antibody to the extracellular domain of VCAM-1 were obtained from R&D Systems, Inc. (Minneapolis, MN). Calibration standards (recombinant proteins), monoclonal anti-human ICAM-1 antibody for detection, and ELISA kits for both sICAM-1 and sVCAM-1 (for the purpose of comparison assays) were also from R&D Systems. Monoclonal anti-human VCAM-1 antibody for detection was from Calbiochem (San Diego, CA). HRP-conjugated sheep anti-mouse-IgG (Amdex) was from Amersham Life Science (Piscataway, NJ). Maxisorp F8 removable well strips were from Nunc (Nunc, Inc., Roskilde, Denmark). Tetramethylbenzidine (TMB) substrate (K-Blue) was from Neogen Corp. (Lexington, KY)

Serum specimens. To establish reference values for sVCAM-1 and sICAM-1 we collected serum specimens from 369 apparently healthy Chinese adults (187 women, 182 men) who visited Chang Gung Memorial Hospital in Taipei, Taiwan, for annual health check-ups. The subjects' ages ranged from 30 to 79 yr. All individuals included in this study had normal clinical chemistry profiles, including normal liver and kidney function tests.

Enzyme-linked immunosorbent assays (ELISAs). The in-house ELISAs for sICAM-1 and sVCAM-1used microtiter plates and followed the sandwich format. They were established by first coating microwells with polyclonal anti-ICAM-1 antibody or anti-VCAM-1 antibody for capturing the analytes. Monoclonal antibodies were used for detection in both ELISAs. The ELISAs involve a one-step procedure in which the serum sample and detecting antibody are added simultaneously into the antibody-coated well. The final color signal in both ELISAs is produced by reacting the bound detecting antibody with Amdex HRP-conjugated sheep anti-mouse-IgG. Because up to 80 molecules of HRP are conjugated in this reagent, the signal is greatly amplified. The ELISA procedures are diagrammed in Fig. 1 and the details are listed in Table 1.

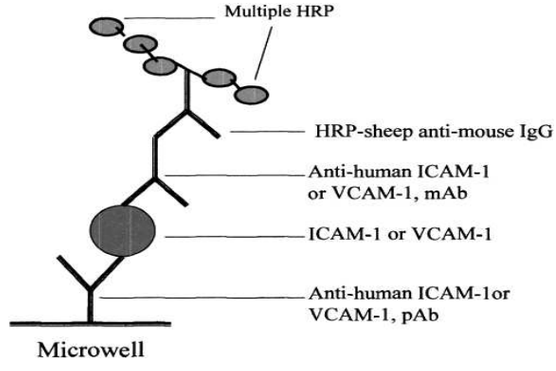

Fig. 1. Diagram of the sandwich format of the in house ELISAs for sICAM-1 and sVCAM-1.

Table 1. Outlines of the in-house ELISAs for sVCAM-1 and sICAM-1.

Analyte	Major Step	Materials	Parameters
sVCAM-1	Coating antibody	Sheep polyclonal antibody to the extracellular domain of human VCAM-1.	100 µl/well, 2 µg/ml, 4°C, overnight
	Sample + detection antibody	10 µl of sample (10x prediluted*) + 90 µl of monoclonal antibody to human VCAM-1.	1 hr, 30°C, (1;20,000)
	Signal antibody	100 µl of Amdex rabbit anti-mouse-IgG-HRP antibody	1 hr, 30°C, (1:10,000)
	Substrate	100 µl of K-Blue solution (tetramethylbenzidine)	10 min, room temperature
	Stop Solution	100 µl of H_2SO_4, 1 mol/L	A 450 nm/620 nm reading†
sICAM-1	Coating antibody	Sheep polyclonal antibody to human ICAM-1	100 µl/well, 1 µg/ml, 4°C, overnight
	Sample + detection antibody	50 µl of sample (20x prediluted*) + 50 µl of monoclonal antibody to human ICAM-1	1 hr, 30°C, (1:1,000)
	Signal antibody	100 µl of Amdex rabbit anti-mouse-IgG-HRP antibody	1 hr, 30°C, (1:2,000)
	Substrate	100 µl of K-Blue solution (tetramethylbenzidine)	10 min, room temperature
	Stop Solution	100 µl of H_2SO_4, 1 mol/L	A 450 nm/620 nm reading†

* Sample prediluted in buffer (phosphate buffer, pH 7.2, 0.01 mol/L, containing bovine serum albumin, 1%, v:v).
† Samples and calibrators were read at 450 and 620 nm (background). The absorbances at 450 nm minus that at 620 nm were used to construct the calibration curves and calculate the results.
Please consult an earlier publication [23] for technical details about ELISA procedures.

Table 2. Characteristics of the ELISAs for sVCAM-1 and sICAM-1.

Analyte	Parameters	Results
sVCAM-1	Concentration range of the calibration curve	0 to 4000 ng/ml
	Assay sensitivity	49 ng/ml
	Assay precision	
	Within-day CV	4.2% (1836 ±77 ng/ml, n = 24) 5.2% (523 ± 27 ng/ml, n = 24)
	Between-day CV	4.1% (1941 ± 80 ng/ml, n = 10) 6.8% (536 ± 37 ng/ml, n = 10)
sICAM-1	Concentration range of the calibration curve	0 to 1000 ng/ml
	Assay sensitivity	40 ng/ml
	Assay precision	
	Within day CV	2.1% (596.8 ± 12.7 ng/ml, n = 24) 2.3% (271 ± 6.4 ng/ml, n = 24)
	Between-day CV	3.3% (659.2 ± 21.6 ng/ml, n = 15) 3.8% (286.2 ± 11.0 ng/ml, n = 15)

Assay sensitivity was determined by assaying serially diluted calibrators at low concentrations until there was no longer a significant decrease in absorbance. Assay sensitivity was defined as the concentration of the diluted calibrator that produced the lowest absorbance.

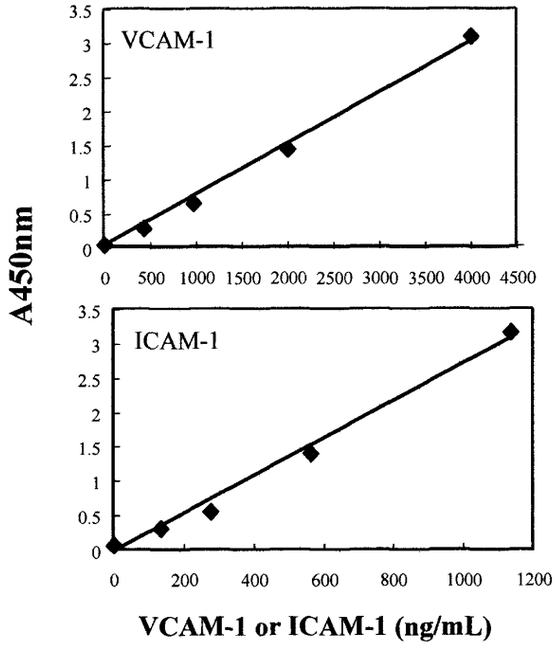

Fig. 2. Calibration curves for ELISAs of serum sVCAM-1 and sICAM-1 concentrations.

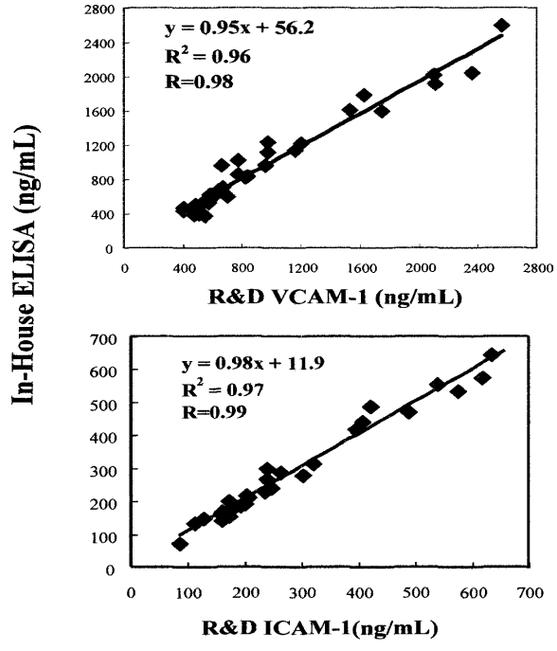

Fig. 3. Comparisons between the results obtained by the in-house ELISAs for sVCAM-1 and sICAM-1 and those obtained by kits obtained from R & D Systems, Inc. (Minneapolis, MN). For sVCAM-1 the serum concentrations ranged from 0 to 2600 ng/ml (n = 34); the SE of the slope was 0.036, the SE of the intercept was 39.98; and the SE of regression was 123.4. For sICAM-1, the serum concentrations ranged from 0 to 700 ng/ml (n = 28); the SE of the slope was 0.031, the SE of the intercept was10.23; and the SE of regression was 25.9.

Table 3. Reference values for sVCAM-1 and sICAM-1 concentrations in serum from apparently healthy Chinese subjects, categorized according to gender and age. Data are listed as mean ± SD and (n).

Age group	Serum sVCAM-1 (ng/ml)		Serum sICAM-1 (ng/ml)	
	women	men	women	men
30 to 39 yr	533 ± 94 (58)	506 ± 108 (37)	238 ± 73 (58)	267 ± 129 (37)
40 to 49 yr	528 ± 175 (38)	496 ± 91 (47)	235 ± 90 (39)	249 ± 79 (47)
50 to 59 yr	550 ± 57 (31)	545 ± 99 (30)	235 ± 78 (30)	259 ± 74 (30)
60 to 69 yr	589 ± 143 (30)	595 ± 174 (36)	262 ± 68 (30)	235 ± 73 (36)
70 yr	683 ± 178 (30)	637 ± 137 (32)	291 ± 95 (30)	230 ± 84 (31)
Overall	568 ± 143 (187)	550 ± 134 (182)	249 ± 82 (187)	248 ± 90 (181)

Results

Assay characteristics. The assay characteristics of the in-house ELISAs are listed in Table 2. The calibration curves established using commercial recombinant proteins from R&D Systems, Inc., covers the concentration range from 0 to 1000 ng/ml for sICAM-1 and from 0 to 4000 ng/ml for sVCAM-1 (Fig. 2). Assay sensitivities were determined to be 40 and 49 ng/ml for sICAM-1 and sVCAM-1, respectively. The coefficients of variation (CVs) for replicate assays (within-day and day-to-day) were all less <10% (Table 2).

Comparison with commercial kits. We compared our in-house developed ELISAs with commercial kits for both sICAM-1 and sVCAM-1from R & D Systems, Inc. (Fig. 3), by paired assays of serum samples. The comparisons gave excellent correlation, with correlation coefficients of 0.99 for sICAM-1 and 0.98 for sVCAM-1.

Reference values. Reference values for sICAM-1 levels were established by assays of serum specimens from 368 apparently healthy Chinese subjects (187 women, 181 men) (Table 3). Serum sICAM-1 levels were not significantly different in men and women. There were no significant differences in serum sICAM-1 levels among the age groups. Serum sICAM-1 levels in the entire group of 369 subjects averaged 249 (SD ± 86) ng/ml.

Reference values for sVCAM-1 levels were established by assays of serum specimens from 369 apparently healthy Chinese subjects (187 women, 182 men) (Table 3). Serum sVCAM-1 levels were not significantly different in men and women. However, serum sVCAM-1 levels were significantly higher in individuals 60 yr old (625 ± 162 ng/ml, n = 128), compared to those <60 yr old (525 ± 110 ng/ml, n = 241; p <0.001.

Discussion

Inflammation is involved in the pathogenesis of many diseases, including cancer, CVD, and type 2 diabetes [1-6]. Proinflammatory cytokines, inflammatory markers, and adhesion molecules are detectable in the serum of these patients. Cytokine-induced adhesion molecules recruit leukocytes to the site of lesions early in the inflammatory process [3,14]. Detecting circulating adhesion molecules has been reported in patients with inflammatory diseases [24,25], various malignancies, heart diseases, and type 2 diabetes [15-21].

Since the patterns of expression of sVCAM-1 and sICAM-1 vary in different diseases, measuring these 2 adhesion molecules simultaneously is likely to achieve higher sensitivity and earlier detection for specific diseases. The in-house ELISAs that are described in this paper have sufficient sensitivity and precision for routine use in clinical laboratories. Both ELISAs involve a one-step procedure that shortens the assay time. The serum specimen and detecting antibody are added to the antibody-coated well simultaneously in order to save an incubation step. The ELISAs can readily be adapted to a microplate autoanalyzer for assays of large numbers of samples.

Increased inflammation in elderly subjects has been reported frequently [26-28]. Therefore, it is not surprising that we found a significantly higher mean value for serum sVCAM-1 levels in apparently healthy persons who were 60 yr old.

Acknowledgement. This study is supported by a research grant from Chang Gung Memorial Hospital (CMRP G32097).

References

1. Nathan C. Points of control in inflammation. Nature 2002;420:846-852.
2. Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420:860-867.
3. Libby P. Inflammation in atherosclerosis. Nature 2002; 420:868-874.
4. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. JAMA 2001;286:327-334.
5. Tak1 PP, Firestein GS. NF-κB: a key role in inflammatory diseases. J Clin Invest 2001;107:7–11.
6. Zetter BR. Adhesion molecules in tumor metastasis. Semin Cancer Biol 1993;4:219-229.
7. Syrigos KN, Salgami E, Karayiannakis AJ, Katirtzoglou N, Sekara E, Roussou P. Prognostic significance of soluble adhesion molecules in Hodgkin's disease. Anticancer Res 2004;24:1243-1247.
8. Green M, Filippou A, Sant G, Theoharides TC.

Expression of intercellular adhesion molecules in the bladder of patients with interstitial cystitis. Urology 2004; 63:688-693.
9. Shin HS, Jung CH, Park HD, Lee SS. The relationship between the serum intercellular adhesion molecule-1 level and the prognosis of the disease in lung cancer. Korean J Intern Med. 2004;19:48-52.
10. Pasieka Z, Kuzdak K, Czyz W, Stepien H, Komorowski J. Soluble intracellular adhesion molecules (sICAM-1, sVCAM-1) in peripheral blood of patients with thyroid cancer. Neoplasma. 2004;51:34-37.
11. Perabo F, Sharma S, Gierer R, Wirger A, Fimmers R, Steiner G, Adam M, Schultze-Seemann W. Circulating intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin in urological malignancies. Indian J Cancer 2001;38:1-7.
12. Ho JW, Poon RT, Tong CS, Fan ST. Clinical significance of serum vascular cell adhesion molecule-1 levels in patients with hepatocellular carcinoma. World J Gastroenterol 2004;10:2014-2018.
13. Qiao XM, Wang ZM. Significance of concentration of serum soluble vascular cell adhesion molecule-1 in epithelial ovarian carcinoma. Ai Zheng 2004;23:81-84.
14. Ross R. Atherosclerosis: an inflammatory disease. NEJM 1999;340:115-126.
15. Carlos TM, Harlan JM. Leukocyte-endothelial cell adhesion molecules. Blood 1994;84:2069-2072.
16. O'Brien KD, McDonald TO, Chait A, Allen MD, Alpers CE. Neovascular expression of E-selectin, intracellular adhesion molecule-1, and vascular cell adhesion molecule-1 in human atherosclerosis and their relation to intimal leukocyte content. Circulation 1996;93:672-682.
17. Price DT, Loscalzo J: Cellular adhesion molecules and atherogenesis. Am J Med 1999;107:85-97.
18. Blankenberg S, Barbaux S, Tiret L. Adhesion molecules and atherosclerosis. Atherosclerosis 2003;170:191-203.
19. Leinonen ES, Hiukka A, Hurt-Camejo E, Wiklund O, Sarna SS, Mattson Hulten L. Low-grade inflammation, endothelial activation and carotid intima-media thickness in type 2 diabetes. J Intern Med 2004;256:119-127.
20. Pickup JC. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. Diabetes Care 2004;27:813-823.
21. Meigs JB, Hu FB, Rifai N, Manson JE. Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. JAMA 2004;291:1978-1986.
22. Iiyama K, Hajra L, Iiyama M, Li H, DiChiara M, Medoff BD, Cybulsky MI. Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation. Circ Res 1999; 85:199-207.
23. Wu TL, Chang PY, Li CC, Tsao KC, Sun CF, Wu JT. Microplate ELISA for urine microalbumin: reference values and results in patients with type 2 diabetes and cardiovascular disease. Ann Clin Lab Sci 2005;35:149-154.
24. Asakura H. Treatment of ulcerative colitis and Crohn's disease with monoclonal antibody. Nippon Rinsho 2002; 60:531-538.
25. Brannigan AE, Watson RW, Beddy D, Hurley H, Fitzpatrick JM, O'Connell PR. Increased adhesion molecule expression in serosal fibroblasts isolated from patients with inflammatory bowel disease is secondary to inflammation. Ann Surg 2002;235:507-511.
26. Krabbe KS, Pedersen M, Bruunsgaard H. Inflammatory mediators in the elderly. Exp Gerontol 2004;39:687-699.
27. Csiszar A, Ungvari Z, Koller A, Edwards JG, Kaley G. Proinflammatory phenotype of coronary arteries promotes endothelial apoptosis in aging. Physiol Genomics 2004; 17:21-30.
27. Lane N. A unifying view of ageing and disease: the double-agent theory. J Theor Biol 2003;225:531-540.