Development of a Microplate ELISA for Circulating E-Selectin: Assay Characterization, Comparison With a Commercial Kit, and Establishment of Normal Reference Values

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E-selectin, an adhesion molecule, is detectable in the blood. Serum levels of E-selectin can be a prognostic indicator for various malignancies. We developed a microplate enzyme-linked immunosorbent assay (ELISA) for the detection of circulating E-selectin by coating the microwell with monoclonal anti-E-selectin. We incubated the specimen and the biotinylated detecting antibody simultaneously, and used HRP-conjugated streptavidin to generate a signal for quantification. The assay has a sensitivity of 3.8 ng/mL, and the coefficients of variation (CVs) for both within-day and day-to-day precision were all <10%. Our in-house-made kit also compared well with a commercial kit from R&D Systems ($r = 0.95$, slope = 0.94). Using our kit we

determined a normal reference value for Chinese individuals of different ages (30–80 years). We found no significant difference between females and males; however, age appeared to have an impact on the normal E-selectin value. We found that normal individuals over 60 years old had higher levels of circulating E-selectin (65.8 ± 19.5 ng/mL, $N = 111$) compared to those under 60 years old (59.5 ± 18.1 ng/mL; $P = 0.002$). Our kit appears to have sufficient sensitivity for detecting elevated circulating E-selectin in various carcinomas. We believe that with the established normal reference value, our in-house-developed ELISA kit is well suited for routine clinical laboratory use. *J. Clin. Lab. Anal.* 17:97–101, 2003. © 2003 Wiley-Liss, Inc.

Key words: E-selectin; adhesion molecule; ELISA; normal reference value; carcinoma

INTRODUCTION

Human E-selectin is an adhesion molecule that is expressed on activated endothelial cells in response to inflammation, which facilitates recruitment of leukocytes to sites of acute inflammation. E-, L-, and P-selectins comprise a group of membrane-attached mosaic proteins. L-selectin is expressed on leukocytes, while E-selectin and P-selectin are expressed on endothelial cells. E-selectin expression on blood vessel wall which keeps leukocytes rolling on the endothelial cell surface (1).

A complex oligosaccharide, known as sialyl Lewis-x, is found on the surface of leucocytes (especially neutrophils). It serves as a ligand for E-selectin, and is responsible for the adhesion of leucocytes to endothelial cells expressing E-selectin. In a similar manner, E-selectin binds to carbohydrate ligands, such as sialyl Lewis A antigen (SLeA) and Lewis X antigen (SLeX), on cancer cells. This not only enhances tumor angiogen-

esis but also promotes the adhesion of tumor cells to endothelial cells at distant sites.

E-selectin and VCAM-1 have been measured in subjects with various malignancies (7, 9). In patients with myeloma, soluble ICAM-1 and VCAM-1 levels were elevated, whereas E-selectin levels actually decreased. Soluble E-selectin (sE-selectin or circulating E-selectin) concentration has been found to be an easily measurable and strong prognostic indicator in node-negative breast cancer patients. High concentrations of circulating E-selectin are associated with reduced overall survival rates. Levels of circulating E-selectin are higher in the presence of liver metastasis. In patients with

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primary breast cancer, high concentrations of circulating E-selectin apparently are associated with reduced tumoral angiogenesis (2,3). Elevated levels of soluble L-selectin and P-selectin were also found in patients with non-Hodgkin's lymphoma and Hodgkin's disease (4), and in ovarian, breast, and GI cancer (5).

It is interesting to note that although several studies have previously demonstrated that the expression of cell surface E-selectin enhances the metastatic process, the shedding of sE-selectin in the circulation has also been considered to be a mechanism of inhibition of tumor progression (6).

We developed a microplate enzyme-linked immunosorbent assay (ELISA) for the detection of circulating soluble E-selectin, with the purpose of studying the role of circulating E-selectin in malignant diseases. We also attempted to establish normal reference values for normal Asian individuals to determine whether race affects normal reference values. At the same time, we also looked into the impact of age and gender on the accurate assessment of circulating E-selectin levels.

MATERIALS AND METHODS

The anti-human E-selectin monoclonal antibody (mAb), the calibrator (a recombinant protein), anti-human E-selectin-biotin conjugated mAb, and the ELISA kit for soluble E-selectin were all purchased from R & D Systems (Abingdon, United Kingdom). Amdex™ streptavidin HRP conjugate was obtained from Amersham Life Science (Arlington, IL). Immulon 4 removable well strips were purchased from Dynatech Laboratories Inc. (Chantilly, VA). The tetramethylbenzidine (TMB) peroxidase substrate and K-blue substrate-TMB were from Neogen Corp. (Lexington, KY).

Normal Serum Sample

To establish normal reference values for circulating E-selectin, we collected serum specimens from normal individuals (30–79 years old) who came to the Chang Gung Memorial Hospital for their annual check-up. The numbers of females and males recruited in this study are listed in Table 1. The majority of the normal specimens were from individuals 40–59 years of age. All individuals selected for this study had normal chemistry profiles, including normal liver and kidney function tests.

Random Serum Specimens From Various Cancers

We also obtained preoperative serum specimens from patients with various forms of cancer, from the serum storage bank of Chang Gung Memorial Hospital, for

TABLE 1. Normal reference values of females and males of various age groups

Age group	Female			Male		
	Mean	SD	n ^a	Mean	SD	n ^a
30–39 y/o	54.3	16.2	40	62.1	15.9	45
40–49 y/o	58.1	18.2	66	61.5	20.5	71
50–59 y/o	57.3	18.3	55	63.6	16.5	37
60–69 y/o	66.5	19.3	41	63.7	19.2	34
≥ 70 y/o	63.8	20.2	18	70.4	20.7	18
Total	59.2	18.6	220	63.2	18.7	205

^an, sample number.

measurements of circulating E-selectin with our in-house-made ELISA kit.

RESULTS

ELISA Development

To develop a microplate ELISA for soluble E-selectin, we coated the microwell with monoclonal anti-E-selectin antibody and detected the captured E-selectin with biotin-conjugated anti-E-selectin monoclonal antibody. To speed up the assay, we added the sample and biotin-conjugated detecting antibody simultaneously into the antibody-coated well. The final signal was provided by Amdex™ streptavidin HRP conjugate. The abbreviated test format is outlined in Table 2.

Assay Characterization

Outlined in Table 3 are the assay characteristics and precision of the in-house-developed ELISA. The calibration curve covers a concentration range of 0–200 ng of E-selectin per milliliter (Fig. 1). The assay sensitivity was determined to be 3.8 ng/mL. Both the assay sensitivity and concentration range covered are practical for assessing circulating E-selectin in the serum. Both within-day and day-to-day precision coefficients of variation (%CV) were also determined to be acceptable for routine use in clinical laboratories.

Normal Reference Values

We determined normal reference values for circulating E-selectin in normal Chinese individuals of various ages (30–70 years old). We examined groups of females and males separately to determine whether there is a gender difference. We found that the serum concentration of E-selectin in males was slightly higher than that in females (males: 63.2 ± 18.7 ng/mL, $N = 205$; females: 59.2 ± 18.6 ng/mL, $N = 220$). The P -value from the independent t -test was 0.03. However, a significant difference in circulating E-selectin was found between

TABLE 2. Outline of the in-house ELISA (one-step)

Major step	Material	Method
Coating Ab	Rat anti human E-selectin, mAb	100 μ L, 2 μ g/mL/well, 4°C overnight
Sample + detection Ab	10 μ L Sample (2X prediluted) + 90 μ L anti-E-selectin-biotin conjugated mAb	1:10,000, 1 hr 30°C
Signal Ab	100 μ L Amdex streptavidin-HRP	1:10,000, 1 hr 30°C
Substrate	100 μ L K-Blue	A450 nm/620 nm, 10 min

TABLE 3. Assay characterization

Concentration range of the calibration curve	0–200 ng/mL
Assay sensitivity	3.8 ng/mL
Assay precision	
Within day	4.8% (50.5 ± 2.4 ng/mL, n = 24) 4.7% (138.8 ± 6.5 ng/mL, n = 24)
Between day	7% (55.8 ± 3.9 ng/mL, n = 10) 6% (132 ± 7.3 ng/mL, n = 10)

Fig. 1. Calibration curve of soluble E-selectin.

individuals (males and females) over 60 years and under 60 years old. For individuals over 60 years old, the mean $\pm$ standard deviation (SD) was 65.8 ± 19.5 ng/mL (N = 111), while it was 59.5 ± 18.1 ng/mL (N = 314) for those under 60 years. The *P*-value was 0.002. We therefore determined the normal cutoff of E-selectin to be 95.7 ng/mL for those under 60, and 104.8 ng/mL for those over 60, respectively, based on a 95% confidence level. The data are presented in Fig. 2 and Table 1.

Comparison With R & D Systems Commercial Kit

We measured circulating E-selectin in a group of serum specimens containing various concentrations of circulating E-selectin using our in-house assay and

Fig. 2. Normal values of soluble E-selectin in both males and females, and in various age groups. **A:** The distribution of normal circulating E-selectin among various age groups is close to a Gaussian distribution. **B:** Normal circulating concentrations of E-selectin in various age groups, and in males and females.

a commercial kit from R & D Systems. The result is shown in Fig. 3. Because we obtained an excellent correlation ($r = 0.95$) with a slope very close to one (slope = 0.94), we believe that the ELISA we developed is acceptable for routine clinical laboratory use. The assay currently is performed manually, which could explain the slight scattering seen in the comparison figure.

E-Selectin in Cancers

To test the sensitivity of our in-house ELISA for circulating E-selectin, we measured circulating E-selectin concentrations in preoperative sera from cancer patients with various carcinomas. The results are shown in Fig. 4. Our kit appears to have sufficient sensitivity to

Fig. 3. Comparison between the in-house-developed ELISA and the R & D Systems commercial kit for circulating E-selectin.

Fig. 4. Concentration of circulating E-selectin detected in various carcinomas. The horizontal line corresponding to the cutoff for a group of normal individuals above the age of 60 is used arbitrarily to indicate how many abnormal levels of circulating E-selectin were found.

detect elevated concentrations of circulating E-selectin in various cancers.

DISCUSSION

Because adhesion is one of the processes that facilitate metastasis, measurements of circulating concentrations of adhesion molecules will provide useful prognostic information for cancer patients (8). Currently used tumor markers, such as CA 15-3, CA 125, and CA 19-9, are only effective for monitoring cancer patients during treatment and for detecting recurrence.

Our in-house microplate assay appears to have sufficient sensitivity and precision to measure circulating E-selectin in cancer patients and aid in prognosis. The sensitivity of this assay (3.8 ng/mL) is far below the

normal cutoff values for any age group of individuals. Although the assay is performed manually, both the within-day and day-to-day precision values are acceptable for routine clinical laboratory use. Because cancer develops most frequently in older individuals, we believed it was important to determine a normal reference value for that age group. Our results showed that age does have an impact on the normal value of circulating E-selectin. In fact, the serum level of circulating E-selectin appears to gradually increase with age. However, the differences between 10-year periods were not significant. For practical reasons, we divided the individuals into two age groups (over 60 years and under 60 years), and a significant difference was found in the level of E-selectin (P -value = 0.002). We suggest that two different cutoffs should be applied to determine whether the serum level of E-selectin is normal or not. The established cutoffs for these two age groups should increase prognostic accuracy for the better management of cancer patients.

On the other hand, the P -value for E-selectin between females and males was found to be 0.03. Although the P -value was <0.03 , indicating a slight difference in the normal level of E-selectin between females and males, we do not see a need to establish cutoffs based on gender. We believe that the difference between the genders is not clinically significant. Creating cutoffs for different genders and as well as different age groups could make the determination of abnormal circulating E-selectin too complicated.

Our normal cutoffs were slightly higher than those listed in the R & D Systems kit (46.3 ± 17.1 ng/mL). It is possible that race was a factor, since we dealt only with Chinese individuals. This may also be related to the fact that we assigned different values to the calibrator and used a different antibody than that employed in the R & D Systems kit.

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