Prognostic Value of Circulating Soluble E-Selectin Concentrations in Node-negative Breast Cancer Patients¹

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ABSTRACT
Several studies have suggested that endothelial cells participate in tumor development. Soluble E-selectin (sE-selectin) is specifically released by activated endothelial cells, and its serum concentration can be considered a marker of endothelial activation. In this study, we assessed the prognostic value of sE-selectin concentrations in node-negative breast cancer patients. Serum sE-selectin concentrations were measured by an ELISA method prior to surgery in 456 node-negative breast cancer patients. We analyzed also tumor size (TS), histoprognostic grading, and steroid hormone receptor status. The mean sE-selectin concentration was 24.9 ± 15.0 ng/ml. The sE-selectin concentrations were mildly correlated with the TS but not with the other factors. For prognostic analyses, the median follow-up was 7.5 years. The cutoff sE-selectin concentration used was 40 ng/ml. In overall survival studies, univariate analyses demonstrated a prognostic value of sE-selectin, TS, and histoprognostic grading, and multivariate analyses demonstrated a prognostic value of sE-selectin and TS. For disease-free survival, univariate and multivariate analyses demonstrated a prognostic value of sE-selectin and TS. sE-selectin concentration is an easily measurable and strong prognostic factor in node-negative breast cancer patients. These results provide further evidence for the role of adhesion molecules expression by endothelial cells in tumor progression.

INTRODUCTION
With improvements in breast cancer screening, >50–60% of tumors are detected early, before axillary nodal involvement (1). These patients are considered to have a better prognosis than node-positive breast cancer patients, but 20–30% of them will relapse after surgery, and there is a need for good prognostic factors to accurately define the subgroup of patients who should benefit from an adjuvant systemic therapy (2, 3). The standard prognostic factors commonly used are TS,³ HPG, and hormone receptor status, but these factors do not allow a precise determination of the patients who will relapse from the disease (3).

Several reports have focused on the interaction between tumor cells and endothelial cells in tumor progression, and some results suggest a role of endothelial cells in angiogenesis and tumor growth (4).

E-selectin is a cell surface adhesion molecule that is specifically expressed by activated endothelial cells (5). E-selectin interacts with the sialyl-Lewisα and the sialyl-Lewisα factors, carbohydrate ligands that are expressed by leukocytes (6). By facilitating leukocyte infiltration, E-selectin is involved in the tissular inflammatory responses. Recent studies have suggested that E-selectin participates in tumor progression and metastasis (7). Initially, experimental studies suggested that E-selectin plays a direct role in the induction of angiogenesis (8, 9). Later, it was shown that tumor cells can, in some cases, abnormally express the above carbohydrate ligands and be attached by activated endothelial cells at distant sites (10).

E-selectin can also be released in a soluble form (sE-selectin) by endothelial cells, and can be easily detected in sera (11). Circulating sE-selectin can be considered a marker of endothelial cell activation.

The aim of this study was to assess the prognostic value of sE-selectin concentrations in patients with node-negative breast cancer.

PATIENTS AND METHODS
Patients. This retrospective study included 456 unselected patients who underwent surgery between April 1986 and April 1992 for node-negative breast cancer in the Center Oscar Lambret (Anticancer Center of the North of France, Lille, France). The mean age of patients was 57.1 years (range, 22–86 years). All of the patients were treated by segmentectomy if the tumor was <3 cm wide and by total mastectomy if the tumor was larger or was centrally located. Axillary dissection was carried out in all cases, and none of the patients had nodal involvement. Surgery was followed by radiation therapy on the chest wall after total mastectomy or on the remaining breast tissue after segmentectomy, and also on the internal mammary, subclavicular, and supraclavicular nodes. Patients with in situ carcinoma were excluded from the study because they did not

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³ The abbreviations used are: TS, tumor size; HPG, histoprognostic grading; sE-selectin, soluble E-selectin; ER, estradiol receptor; PgR, progesterone receptor; RR, relative risk; CI, confidence interval.

1 This work was supported by a grant from the Ligue Nationale Contre le Cancer (Paris, France). The mean age of patients was 57.1 years (range, 22–86 years). All of the patients were treated by segmentectomy if the tumor was <3 cm wide and by total mastectomy if the tumor was larger or was centrally located. Axillary dissection was carried out in all cases, and none of the patients had nodal involvement. Surgery was followed by radiation therapy on the chest wall after total mastectomy or on the remaining breast tissue after segmentectomy, and also on the internal mammary, subclavicular, and supraclavicular nodes. Patients with in situ carcinoma were excluded from the study because they did not
receive adjuvant radiation therapy after surgery. Prior to 1993, our policy was to not perform adjuvant systemic therapy in node-negative patients, and none of the patients included in this study received adjuvant chemotherapy or hormone therapy. Consequently, all patients included in this study had the same initial treatment. Prior to surgery, all of the patients had chest radiograms, liver ultrasonography, and bone scanning. None of the patients had evidence of distant metastasis. To avoid a possible increase in sE-selectin concentrations associated with impaired liver or kidney functions, we verified that all patients had normal liver (serum bilirubin, ≤30 μmol/liter, aspartate transaminase, ≤25 IU/ml) and renal (serum creatinine, ≤130 μmol/liter) functions. For the same reason, in patients who had a breast diagnostic biopsy prior to surgery, the serum sample was drawn at least 2 weeks after the biopsy.

In the studied population, the median follow-up duration of living patients was 7.5 years. Within this population, the number of deaths was 111 (24.3%), and the number of relapses was 120 (26.3%).

Pathology. After surgery, the tumor samples were divided into two parts: one part was frozen for hormone receptor analysis, and the other was submitted for histological examination. Tumor samples consisted solely of invasive adenocarcinomas. The HPG was obtained using the Scarff and Bloom criteria, as described previously (12). We analyzed also the pathological subtypes. Pathological subtypes were divided into three categories: ductal type, lobular type, and other types.

ER and PgR Assays. Both ERs and PgRs were determined by the dextran-coated charcoal method, as described previously (13). For preparation of cytosols, the frozen tissues were weighed and then pulverized. The tissues were homogenized in 20 mM Tris, 3 mM EDTA, 1 mM DTT, 0.01% azide, and 0.01 mM sodium molybdate (pH 7.6). The homogenate was centrifuged at 800g for 10 min, and the supernatant was ultracentrifuged at 105,000g for 60 min. Concentrations higher than 10 fmol/mg protein were considered positive. Our laboratory is affiliated with the European Organization for Research and Treatment of Cancer Receptor Study Group, which is involved in quality control of the assays (14).

sE-Selectin Assay. Serum samples were obtained from the patients prior to surgery. Five ml of blood were collected on EDTA and were centrifuged at 3000g for 10 min. Sera were stored at –20°C until analysis. Concentrations of sE-selectin were measured in duplicate using a commercial ELISA kit (R&D Systems, Abington, United Kingdom). The performance characteristics of the assay were specified by R&D Systems. A typical sensitivity of the ELISA kit used in this study is <2 ng/ml, which is sufficient to detect minimal physiological concentrations. For a mean value of 50 ng/ml, the intra-assay variability was 4.7%, and the interassay variability was 5.6%. Briefly, microtiter ELISA plates coated with a specific capture monoclonal antibody were used. Standards and samples were added to the plate and then incubated for 1.5 h at room temperature. After washing, the bound sE-selectin was detected by incubation with a specific antibody conjugated to the enzyme horseradish peroxidase. After removal of unbound material by aspiration and washing, the amount of conjugates bound to the well was detected by reaction with a substrate (tetramethylbenzidine). The reaction was stopped by the addition of 1 mM HCl, and the A450 was measured.

Statistical Analyses. Associations between parameters were assessed using the Spearman’s rank and the Mann-Whitney U nonparametric tests. For the prognosis analyses, the sE-selectin concentration was entered as a dichotomous variable after selecting a single cutoff value that allowed maximal separation between groups at low or high risk for relapse and/or death. To find the optimum cutoff value, we analyzed the following six values: median, first quartile, third quartile, mean, mean + 1 SD, and mean + 2 SD. The sE-selectin concentrations were also tested as continuous variables. Using the same approach, we tested the following cutoff values for TS: <3 cm versus ≥3 cm; <2 cm versus ≥2 cm; <2 cm versus >2 cm; and 5 cm versus ≥5 cm. TS was also tested as a continuous variable. HPG, ER, and PgR were entered as dichotomous variables (grades I–II versus III, positive versus negative, and positive versus negative, respectively). All of the analyzed covariates were analyzed in the univariate and the multivariate analyses.

Statistical analyses were carried out using SAS statistical software on a VAX VMS 6320. Overall survival and relapse-free survival curves were calculated using the Kaplan–Meier method. Comparison between curves was carried out by the log-rank test. The proportional hazards regression method of Cox (15) was used to assess the prognostic significance of parameters taken in association. No time-dependent variable was introduced. The latter analyses were performed with Dash Software (Dash Software Development Group, Boston, MA).

RESULTS

Pathological Features. HPG was obtained in 437 of 456 cases (95.8%). It was grade I in 63 (14.4%), grade II in 218 (49.8%), and grade III in 156 (35.6%) of the 437 cases analyzed. ER and PgR were obtained in 441 of 456 cases (96.7%). ER and PgR positivity was found in 315 (71.4%) and 297 (67.3%), respectively, of the 441 cases analyzed.

For the pathological subtype, 347 of 456 patients (76.0%) had ductal carcinoma, 57 of 456 (12.5%) had lobular carcinoma, and 52 of 456 (11.5%) had other types.

Correlations between sE-Selectin Concentrations and Other Parameters. The mean concentration of circulating sE-selectin was 24.9 ± 15.0 ng/ml (mean ± SD; range, 2–111 ng/ml). Using the Spearman’s rank correlation test, sE-selectin concentration and TS were mildly correlated (r = 0.01, r = 0.12). Using the Mann-Whitney U test, we found no relation between sE-selectin concentrations and HPG (P = 0.23), ER (P = 0.89), and PgR (P = 0.14) status. The mean concentrations of sE-selectin were 26.4 ± 12.6 ng/ml in patients with ductal carcinoma, 29.1 ± 12.9 ng/ml in patients with lobular carcinoma, and 26.7 ± 15.2 ng/ml in patients with another pathological type. Using the Student’s t-test, we found no significant difference between these values.

Prognosis Analyses. For sE-selectin concentrations, a prognostic value was found for two cutoff values: third quartile (33 ng/ml) and mean + 1 SD (40 ng/ml). A prognostic value was also found when sE-selectin concentration was tested as a continuous variable. The optimum cutoff value was the mean +
1 SD, and it was retained for the following analyses. Concentrations of $>40$ ng/ml were found in 60 of 456 cases (13.2%).

For TS, the 3-cm cutoff was associated with a significant prognostic value. No prognostic value was found for other cutoffs or when TS was tested as a continuous variable. TS was $\leq 3$ cm in 333 of 456 patients (73.0%) and $\geq 3$ cm in 123 of 456 patients (26.9%).

The univariate analysis of overall survival showed the prognostic value of sE-selectin concentrations ($P = 0.001$; Table 1 and Fig. 1), TS ($P = 0.005$), and HPG ($P = 0.03$). The univariate analysis of disease-free survival showed the prognostic value of sE-selectin ($P < 0.0001$; Fig. 2) and TS ($P = 0.01$).

In the multivariate analysis, a prognostic value for overall survival was found for sE-selectin concentrations ($P = 0.05$, RR = 1.73, 95% CI = 1.00–3.01) and TS ($P = 0.02$, RR = 1.74, 95% CI = 1.10–2.75). A prognostic value for disease-free survival was found for sE-selectin concentrations ($P = 0.003$, RR = 2.25, 95% CI = 1.33–3.82), and TS ($P = 0.01$, RR = 1.82, 95% CI = 1.15–2.85; Table 1).

Because sE-selectin concentrations and TS had a prognostic value, we divided the patients into four groups, as follows: group A, sE-selectin, $\leq 40$ ng/ml, and TS, $<3$ cm ($n = 298$); group B, sE-selectin, $\leq 40$ ng/ml, and TS, $\geq 3$ cm ($n = 98$); group C, sE-selectin, $>40$ ng/ml, and TS, $<3$ cm ($n = 34$); and group D, sE-selectin, $>40$ ng/ml, and TS, $\geq 3$ cm ($group D, n = 26$). The overall survival was significantly shorter in group D patients compared to group C patients ($P = 0.04$), group B patients ($P = 0.04$), and group A patients ($P = 0.01$; Fig. 3). The relapse-free survival was significantly shorter in group D

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall survival</th>
<th>Disease-free survival</th>
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<tbody>
<tr>
<td></td>
<td>Univariate (risk ratio, 95% CI)</td>
<td>Multivariate (risk ratio, 95% CI)</td>
</tr>
<tr>
<td>Serum sE-selectin concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous variable $\leq 40$ vs. $&gt;40$ ng/ml ($n = 396$ vs. $n = 60$)</td>
<td>0.01 (—)</td>
<td>0.03 (—)</td>
</tr>
<tr>
<td>Continuous variable $&lt;3$ cm vs. $\geq 3$ cm ($n = 318$ vs. $n = 112$)</td>
<td>0.12</td>
<td>0.29</td>
</tr>
<tr>
<td>Grades I–II vs. grade III ($n = 251$ vs. $n = 144$)</td>
<td>0.03 (1.53, 1.04–2.27)</td>
<td>0.49 (1.17, 0.73–1.93)</td>
</tr>
<tr>
<td>ER status Positive vs. negative ($n = 311$ vs. $n = 125$)</td>
<td>0.81</td>
<td>0.58 (0.83, 0.49–1.57)</td>
</tr>
<tr>
<td>PgR status Positive vs. negative ($n = 293$ vs. $n = 143$)</td>
<td>0.26</td>
<td>0.73 (1.10, 0.63–1.94)</td>
</tr>
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Fig. 1 Univariate analysis of the overall survival according to sE-selectin concentration status. The sE-selectin concentration used as cutoff was 40 ng/ml (mean + 1 SD).
patients compared to group C patients ($P = 0.03$), group B patients ($P = 0.04$), and group A patients ($P = 0.001$; Fig. 4). The numbers of deaths and relapses are presented in Table 2.

DISCUSSION

Traditional prognostic factors are not sufficient to precisely predict the risk of relapse in patients with node-negative breast cancer, and several other parameters have been studied. The assessment of tumor cell proliferation rate by the S-phase fraction (by measurement of DNA precursors into newly synthesized DNA or by immunohistochemical methods) yields useful information about prognosis, but it requires additional time, technical expertise, and laboratory expense (16). S phase and ploidy can be evaluated by flow cytometric analysis, but their prognostic value for this method remains controversial in breast cancer (17). The prognostic value of tumor angiogenesis, assessed by the tumor microvessel count, remains controversial, but this probably results from methodological differences between the studies; this methodology needs standardization (18–20). In a previous study, we found that the level of tumor urokinase-type plasminogen activator had an independent prognostic value for relapse-free survival in node-negative breast cancer patients and that this prognostic value was higher than that of p53 expression (21). In another study, we found that serum circulating anti-p53 antibodies were detectable in 12% of patients with locoregional breast cancer and that these antibo-
dies were associated with a worse outcome (22). However, in that study, the prognostic value of anti-p53 antibodies was only found in patients with node-positive breast cancers.

In this study, circulating sE-selectin concentration was an important prognostic factor for overall and disease-free survivals in patients with node-negative breast cancer, and this prognostic value was higher than that of traditional parameters. TS had also a prognostic value. When sE-selectin concentrations and TS were entered as continuous variables, the results were similar, and prognostic value of sE-selectin concentrations remained higher than that of TS (data not shown). The HPG had a prognostic value only in univariate analysis of relapse-free survival. The lack of prognostic significance of ER and PgR may allow us to better focus treatment efforts on those patients at high risk of relapse and to avoid toxic effects of treatment in patients at low risk of relapse.

Measurement of sE-selectin concentrations can be rapidly and easily performed in serum or plasma of patients and can provide a prognostic estimate prior to surgical therapy. Yet, the sE-selectin cutoff value used here (mean + 1 SD) was the optimum cutoff in our statistical analyses, and a laboratory standardization must be performed to define the optimum cutoff value of sE-selectin in each laboratory.

A high concentration of sE-selectin can be considered a marker of endothelial cell activation, and this provides further evidence for a relationship between endothelial cell activation and tumor development, as outlined below.

(a) We could suppose that the prognostic value of endothelial cell activation is related to angiogenesis, and it has been also suggested, in some experimental studies, that cell surface E-selectin is able to directly enhance angiogenesis (8, 9). Yet, the relationship of angiogenesis to E-selectin remains controversial. Gerritsen et al. (24) found that angiogenesis was possible in E-selectin-deficient mice. The angiogenesis inhibitors angiotatin and AGM-1470 have been found to increase endothelial E-selectin expression (25, 26). Conversely, the induction of angiogenesis by tumor-derived basic fibroblast growth factor or vascular endothelial growth factor is associated with a decreased E-selectin expression by tumor-associated endothelial cells (27). In a previous study, we also found a negative correlation between circulating sE-selectin concentrations and tumor angiogenesis in patients with breast cancer (28). These results suggest that E-selectin is not required for tumor angiogenesis and that there are at least two endothelial cell activation states. One state is characterized by endothelial cell proliferation and leads to angiogenesis. The other activation state is based on adhesion molecules expression and allows communications with

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**Table 2** Numbers of relapses and deaths in four groups of patients

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of relapses (%)</th>
<th>No. of deaths (%)</th>
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<tbody>
<tr>
<td>A (n = 288)</td>
<td>60 (20.8)</td>
<td>58 (20.1)</td>
</tr>
<tr>
<td>B (n = 98)</td>
<td>31 (31.6)</td>
<td>29 (29.5)</td>
</tr>
<tr>
<td>C (n = 34)</td>
<td>13 (38.2)</td>
<td>12 (35.2)</td>
</tr>
<tr>
<td>D (n = 26)</td>
<td>16 (61.5)</td>
<td>12 (46.1)</td>
</tr>
</tbody>
</table>

*The groups were according to sE-selectin concentration and TS, as follows: group A, sE-selectin, ≤40 ng/ml, and TS, <3 cm; Group B, sE-selectin, ≤40 ng/ml, and TS, ≥3 cm; group C, sE-selectin, >40 ng/ml, and TS, <3 cm; group D, sE-selectin, >40 ng/ml, and TS, ≥3 cm.*
other cells. The above results suggest that these two activation states are distinct processes.

(b) A second explanation for the relationship between endothelial cell E-selectin expression and tumor development is that endothelial cell E-selectin expression may promote tumor metastasis. In some cases, tumor cells are, indeed, able to express E-selectin carbohydrate ligands and can be attached by endothelial cells in distant sites (7). In a recent study, we assessed the expression of five sialyltransferases in breast primary tumors, and we frequently found a high expression of the ST3Gal III, an enzyme involved in the sialyl-Lewis\(^a\) factor synthesis (29). High ST3Gal III expression was associated with a poor prognosis. This may result from the adhesion of circulating tumor cells expressing the sialyl-Lewis\(^a\) factor to endothelial cells expressing E-selectin.

(c) A direct participation of endothelial cells in tumor development cannot be excluded. Some studies have, indeed, demonstrated that endothelial cells can directly stimulate tumor cells by releasing important paracrine growth factors for tumor cells (e.g., basic fibroblast growth factor, insulin growth factor-2, platelet-derived growth factor, and colony-stimulating factors; Refs. 4, 30, and 31).

The cause of the endothelial cell E-selectin expression in patients with breast cancer remains unclear. It may result from a tumor-mediated stimulation. Tumor cells can, in some cases, secrete interleukin 1 or tumor necrosis factor-\(\alpha\), which are potent endothelial activators and inducers of E-selectin expression and release (32, 33). In a recent study, Nguyen et al. (34) found that breast tumor-derived interleukin 1 increased endothelial E-selectin expression.

The role of endothelial cells activation in tumor development suggests a potential benefit from therapeutic endothelial cell inhibition. Several new agents are under investigation, especially antiangiogenic agents. It would be important to assess also the potent inhibition of endothelial cell adhesion molecules expression induced by these agents, and the monitoring of circulating sE-selectin concentrations may be helpful in this way.

In conclusion, we showed, in a large series of patients with node-negative breast cancer, that concentrations of circulating sE-selectin have a strong prognostic value for overall and disease-free survivals. These results suggest a relationship between endothelial cell adhesion molecules expression and tumor progression.

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