Pathologic evaluation of tumor-associated macrophage density and vessel inflammation in invasive breast carcinomas

Yoshihiro Morita
University of Oklahoma Health Sciences Center

Roy Zhang
University of Oklahoma Health Sciences Center

Macall Leslie
University of Oklahoma Health Sciences Center

Smita Adhikari
University of Oklahoma Health Sciences Center

Nafis Hasan
Thomas Jefferson University

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Authors
Yoshihiro Morita, Roy Zhang, Macall Leslie, Smita Adhikari, Nafis Hasan, Inna Chervoneva, Hallgeir Rui, and Takemi Tanaka
Pathologic evaluation of tumor-associated macrophage density
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YOSHIHIRO MORITA1*, ROY ZHANG2*, MACALL LESLIE3, SMITA ADHIKARI3, NAFIS HASAN4,
INNA CHERVONEVA5, HALLGEIR RUI6 and TAKEMI TANAKA1,2

1Stephenson Cancer Center, 2Department of Pathology, School of Medicine, 3Department of Biostatistics and Epidemiology,
College of Public Health, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104; Departments of
4Pharmaceutical Sciences and 5Pharmacology and Experimental Therapeutics, Thomas Jefferson University,
Philadelphia, PA 19107; 6Department of Pathology, Medical College of Wisconsin Cancer Center, Milwaukee, WI 53226, USA

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Abstract. Tumor-associated macrophages (TAMs) are major constituents of the tumor microenvironment in solid tumors
and have been implicated as mediators of tumor progression, invasion and metastasis. Correspondingly, accumulation of
TAMs is associated with unfavorable clinical outcomes in numerous types of solid tumors. E-selectin is a hallmark of
inflammation and a key adhesion molecule that accommodates the initial contact of circulating immune cells with the inflamed
vessel surface. Currently, the association between E-selectin and TAMs is not fully elucidated; therefore, the present study
investigated the association between vessel inflammation, TAM infiltration, and clinical outcome in breast cancer. A
total of 53 procedure-naive invasive breast cancer cases were immunohistochemically analyzed for the presence of cluster
of differentiation (CD)68+ TAMs, E-selectin+ vessels and tumor inflammation. The association between CD68 and E-selectin
expression, and tumor inflammation as well as overall survival was evaluated using Kaplan-Meier survival curves and multi-
variable Cox's proportional hazards regression analysis. The abundance of TAMs was identified to be positively associated
with tumor inflammation, estrogen receptor and E-selectin expression, and tumor inflammation as well as overall survival. The abundance of TAMs
was associated with the presence of E-selectin-expressing inflamed tumor vessels and tumor inflammation, as well as
overall survival in patients with invasive breast carcinoma.

Introduction

Inflammation is a hallmark of cancer, not only triggering
tumor development, but also promoting tumor progression,
therapy resistance and metastasis (1,2). Breast cancer is a
common type of malignancy, with >1.67 million new cases
and 522,000 mortalities reported in 2012 worldwide (3).
Approximately 30% of females with breast cancer experience
recurrence within 5 years, with a 50% chance of developing
distant metastases (4,5). Cancer is aptly described as a wound
that never heals since solid tumors are chronically inflamed
and immune cells in concert with other stromal components
influence cancer cell behavior. Immune infiltrates represent a
significant component of the mass in solid tumors (6) and aid
in regulating cancer cell growth, angiogenesis and invasion
via the production of an array of cytokines, reactive oxygen
species, and proteases (7-10). Tumor-associated macrophages
(TAMs) are a prominent component, serving a central role in
promoting tumor growth and metastasis (1). Accordingly, a
greater abundance of TAMs has been associated with metastas-
tis and poor prognosis in numerous types of solid tumors
including breast (11,12), lung (13,14), prostate (15), colorectal,
and pancreatic cancer (16-19). TAMs are recognized as
potent producers of growth factors (transforming growth
factor-β, fibroblast growth factors and epidermal growth
factor), pro-angiogenic factors [vascular endothelial growth
factor, tumor necrosis factor-α (TNF-α), interleukin (IL)-8,
matrix metalloproteinase and platelet derived growth factor],
proteases (cathepsin and serine proteases) and cytokines
(IL-10), which profoundly affect epithelial cancer cell growth,
angiogenesis, local invasion, extracellular matrix degrada-
tion, epithelial-mesenchymal transition, metastasis, therapy
response, and immunosuppression (20-22).

Macrophages originate from peripheral blood mono-
nuclear cells derived from bone marrow and are recruited into
the tumor via colony stimulating factor 1 and C-X-C motif
chemokine ligand 12, released from cancer cells or the tumor

Correspondence to: Dr Takemi Tanaka, Stephenson Cancer
Center, University of Oklahoma Health Sciences Center, Room 1415,
975 NE 10th, BRC-W, Oklahoma City, OK 73104, USA
E-mail: takemi-tanaka@ouhsc.edu

*Contributed equally

Key words: E-selectin, cluster of differentiation 68, tumor-
associated macrophages, breast cancer, inflammation, overall
survival, immunohistochemistry
microenvironment (23). For successful tissue migration, circulating immune cells undergo a sequential multistep adhesion cascade initiated by adhesion to the vessel surface (24-28). Vascular expression of selectin family member proteins aids physical interaction with counter-receptor ligands expressed on immune cells, including sialyl Lewis^x (sLe^x), sialyl Lewis^a (sLe^a), cluster of differentiation (CD)44, cutaneous lymphocyte-associated antigen and P-selectin glycoprotein ligand-1 (29-31). E-selectin (also known as CD62E, endothelial cell leukocyte adhesion-1 or Leukocyte-endothelial cell adhesion molecule 2) is exclusively expressed on the luminal surface of inflamed vessels and serves a role in the catch bond that switches from rolling adhesion to integrin-mediated firm adhesion (32). Thus, elevated vascular E-selectin expression levels have been reported in a range of solid tumors, including breast (11,12), lung (13,14) and pancreatic (16,18) cancer. E-selectin expression often synchronizes with an abundance of sLe^a- or sLe^x-positive immune infiltrates in the tumor (33,34). The present study investigated the abundance of tumor vascular E-selectin and macrophage marker CD68 expression levels in order to understand the association between inflamed tumor vessels and TAM infiltration, as well as their role in breast cancer prognosis.

Materials and methods

Tumors. Surgical whole mounts from a total of 100 human breast carcinoma specimens from females diagnosed between January 1987 and December 1988 from the pathology archives at Thomas Jefferson University (Philadelphia, USA) were used in the present study. The average age of patients at the time of surgery was 61.8±16.4 years. Cases with tissue containing ductal carcinoma in situ (DCIS) only, inflammatory breast cancer or other concurrent malignancies were excluded from the present study. Cases with no reactivity to vimentin staining were eliminated from the study. Only cases with fully annotated information regarding demographics, estrogen receptor status and then incubated with mouse anti-CD68 monoclonal antibody at 1:25 (cat. no. M0876; Dako) overnight at 4°C. Following a brief wash with PBS, the slides were incubated with pre-dilute secondary alkaline phosphatase-polymer conjugated MACH2 anti-mouse IgG (cat. no. MALP521; Biocare Medical LLC) for 1 h at room temperature and then visualized using Fast-Red (Biocare Medical LLC) for 7 min, followed by counterstain with Mayer Hematoxylin (Dako) for 4 min both at room temperature. The slides were air-dried and mounted. As a negative control, breast carcinoma tissues were immunostained with the secondary IgG only.

Pathologic evaluation. All immunohistochemically stained slides were evaluated by a board certified surgical pathologist (Department of Pathology, School of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA) for breast pathology. The tumors were classified and graded according to the protocol from of the College of American Pathologists (Protocol for the Examination of Specimens from Patients with Invasive Carcinoma of the Breast according to InvasiveBreast 3.3.0.0.) (35). Tumor inflammation was defined as positive or negative, as characterized by the presence of lymphocyte clusters. Immunohistochemical reaction to E-selectin was graded using intensity as a score of 0, 1+, 2+ or 3+ for no, weak, moderate or strong reaction in endothelial cells within the tumor, respectively. Immunohistochemical staining of CD68 was quantitatively categorized as a score of 0, 1+, 2+ or 3+ for no CD68^+ cells, ≤10 CD68^+ cells, 11-20 CD68^+ cells or ≥21 CD68^+ cells in the observing field at x200 magnification within the tumor, respectively. All images were viewed under a light microscope (DM2500; Leica, Buffalo Grove, IL, USA) and images were captured using digital cooling color camera (DFC450; Leica).

Statistical analysis. The Fisher's exact test was used to analyze the association between age and tumor pathological parameters using CD68 and E-selectin expression levels. Spearman's rho was used to determine the correlation between CD68^+ TAMs, and E-selectin expression level and tumor inflammation. The Kaplan-Meier method was used to estimate OS as a function of time, and differences were analyzed using the log-rank test. Cox proportional hazards regression analysis was used for multivariable analysis of prognostic factors in relation to OS. The statistical software SAS, version 9.4 (SAS Institute Inc., Cary, NC, USA) was used to perform statistical analyses. GraphPad Prism version 6 (Graphpad Software, Inc., La Jolla, CA, USA) was used to generate Kaplan-Meier curves. P<0.05 was considered to indicate a statistically significant difference.
Fig. 1

Fig. 2

Table I. Association between clinicopathologic parameters, and CD68 and E-selectin expression in procedure-naïve invasive breast carcinoma tissues.

<table>
<thead>
<tr>
<th>Clinicopathologic parameters</th>
<th>Total no. cases</th>
<th>CD68 expression</th>
<th>E-selectin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>All cases</td>
<td>53</td>
<td>34</td>
<td>19</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤60</td>
<td>20</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>&gt;60</td>
<td>33</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>Tumor inflammation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)</td>
<td>25</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>(+)</td>
<td>28</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Nottingham histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I</td>
<td>3</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Grade II + III</td>
<td>50</td>
<td>26</td>
<td>15</td>
</tr>
<tr>
<td>ER status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>11</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Positive</td>
<td>42</td>
<td>30</td>
<td>12</td>
</tr>
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</table>

*Statistically significant difference following Fisher's exact probability test. CD68, cluster of differentiation 68.

Results

Tumor characteristics. Following exclusions, a total of 53 invasive breast cancer cases that had been first-time diagnosed by surgical resection in 1986-1988 at Thomas Jefferson University were used in the present study. Tumor characteristics of these patients are presented in Table I. Cases were categorized as either high (score 3+) or low (score 0-2+) CD68 expression level according to the abundance of CD68+ TAMs in the tumor core and high (score 3+), or low (score 0-2+) expression level of E-selectin on vessels in the tumor area. CD68+ TAMs were significantly associated with tumor inflammation (P=0.005) and ER status (P=0.037). E-selectin expression level was associated with age (P=0.016) and was significantly higher among females over the age of 60 years (Table I).

Pattern of CD68+ TAM infiltration and E-selectin+ vessel inflammation in breast tumors. Representative images of high and low expression levels of CD68+ TAMs, and E-selectin+ inflamed vessels are presented in Fig. 1. Various expression levels of CD68+ TAMs were consistently present in the tumor stroma and core of all 53 cases (Fig. 1A-C). Considerable, multilayered CD68+ TAM deposition in the necrotic area of the tumors was observed. CD68+ TAMs were also abundant in the mammary fat adjacent to the tumor, as well as around the luminal surface of vessels (Fig. 1C). E-selectin was predominantly expressed on vessels within the tumor stroma (Fig. 1D and E). No positive signal for E-selectin was detected in other tumor components, including cancer cells, fibroblasts, immune infiltrates or the apical side of the vessels. The size of E-selectin-expressing vessels varied from small capillaries to large vessels in the stroma and fat adjacent to the invasive front of tumors. Vessel structure was well retained within the stroma but often compressed, crushed or even absent in the tumor core. Overall, 88.7% of the breast carcinoma cases exhibited E-selectin expression on their tumor vessels. Consistent with inflammation of the adipose tissue adjacent to the tumor, E-selectin expression level was also high in vessels of the neighboring peripheral adipose tissue. Of note, vascular E-selectin expression level was present in the stroma surrounding the mammary duct in invasive carcinomas that retained a ductal structure (Fig. 1F). A similar pattern of E-selectin expressing vessels in the stroma around the mammary duct in DCIS only cases was also revealed (data not shown), suggesting the presence of peritumoral inflammation at the pre-invasive stage.

Association between vessel inflammation and TAM infiltration. Double immunohistochemistry for CD68 (pink) and E-selectin (brown) was performed to evaluate their association. CD68+ TAMs were abundant in close proximity to E-selectin-expressing vessels in the tumor stroma and peripheral tissue adjacent to the tumor (Fig. 2A and B). CD68+ TAMs were sparsely present in carcinoma cell rich areas; however, E-selectin expression was limited to the surrounding inflamed area and absent or weakly present in the tumor core (Fig. 2B). CD68+ TAMs were also highly abundant in necrotic areas, but E-selectin was absent within and adjacent to the necrotic core (Fig. 2C). TAMs and E-selectin were present at the location where the ductal structure was retained (Fig. 2D). Association between the abundance of CD68+ TAMs and E-selectin+ vessels was evaluated using a 4-level scoring scale (0, 1+, 2+, 3+) for the expression level of each marker. High abundance of CD68+ TAMs and E-selectin+ vessels (3+/3+) was demonstrated in 7.5% of the overall analyzed samples. CD68+ TAMs and E-selectin expression levels were positively correlated (r=0.30, P=0.030; Table II). Additionally, CD68+ TAMs were...
significantly correlated with tumor inflammation ($r=0.54$, $P=0.001$; Table II).

**Abundance of markers and clinical outcome.** OS was determined and graphically presented using the Kaplan-Meier method, and a Cox proportional hazards regression model was used for multivariable analysis of the association between clinicopathological parameters and marker expression level with OS. Tumor inflammation was significantly associated with OS among patients with breast cancer [hazard ratio...
Tissue infiltration by circulating leukocytes occurs in response to tissue damage and injury. In the context of solid tumors, cell death arises from intrinsic and extrinsic inducers, initiating an inflammatory cascade in an attempt to scavenge debris, and repair damaged tissue. Intrinsic cell death is hypoxia-derived necrosis or DNA-damage-associated apoptosis, whereas extrinsic cell death is associated with external stimuli, including chemotherapy, biopsy, surgery or radiation therapy. The standard of care for breast cancer has altered significantly over the past 3 decades (35). Diagnostic needle biopsy became popular in the 1990s (36) and neoadjuvant chemotherapy for relatively large, locally advanced tumors emerged in the 2000s (37). Both procedures provoke inflammation accompanied by cell death in the tumor and neighboring peripheral tissue. Thus, it is likely that recent surgically resected tumors contain inflammation induced by extrinsic stress along with naturally occurring intrinsic cell death. Although external stress naïve tumors can be analyzed using biopsy samples, such samples contain only small and limited amounts of tissue, making the capture of the overall tumor environment difficult. Analysis of naïve tumors collected by excisional biopsy essentially eliminates locally advanced tumors since this method is typically only used in early stage small-sized tumors. Thus, in order to understand the association of vessel inflammation and TAM infiltration in intrinsic tumor inflammation, the present study specifically targeted surgically resected, procedure-naïve breast tumor tissue samples collected between 1986 and 1988, when needle biopsy was not yet broadly adopted as the standard of care.

The involvement of E-selectin in cancer has long been recognized, as evidenced by histopathological studies; however, its clinical implications have been controversial (11,12,38). The results of the present study revealed that 88.7% of procedure-naïve breast tumors expressed E-selectin in the vessels within the tumor. Previously, the prevalence of E-selectin-positive vessels has been reported as 55.7% (n=113) (11) and 77.6% (n=22) (12) in frozen breast tumor tissue sections. A previous study by Charpin et al (11) demonstrated a positive association between E-selectin expression level, and vascular cell adhesion molecule-1, very late antigen-2 and CD44 expression levels, and a negative association with E-cadherin expression level. However, the latter was positively associated with ER-negative breast cancer, which may be due to the release of higher expression levels of IL-1 and TNF-α from ER-negative compared with ER-positive breast cancer cells. The present study and the study by Charpin et al (11) did not determine an association between E-selectin expression and ER status, but both studies identified an association between E-selectin, and CD68+ TAMs or CD44+ immune infiltrates, a common marker for immune cells.

TAMs are classified as either pro-inflammatory M1 or pro-tumorigenic M2 macrophages (36), although it is yet to be determined which of these is more clinically important for prognosis (37,39). Unlike T-lymphocytes, whose phenotypes are classified by differentiation, the TAM phenotype is plastic and determined by its surrounding microenvironment (40). For example, the M1 phenotype can switch to M2 in response to T helper 2-released cytokines, including IL-13 and IL-4 (41). Accordingly, the overall composition and

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazard ratio</th>
<th>95% CI</th>
</tr>
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<tbody>
<tr>
<td>Tumor inflammation (ref. positive)</td>
<td>0.326</td>
<td>1.48</td>
</tr>
<tr>
<td>ER status (ref. negative)</td>
<td>0.341</td>
<td>1.50</td>
</tr>
<tr>
<td>E-selectin expression (ref. high)</td>
<td>0.112</td>
<td>0.46</td>
</tr>
<tr>
<td>CD68 expression (ref. high)</td>
<td>0.045*</td>
<td>2.34</td>
</tr>
</tbody>
</table>

*Statistically significant. CI, confidence interval; ER, estrogen receptor; CD68, cluster of differentiation.
balance of immune subsets determines the pro-tumorigenic potential and fate of the tumor. TAMs were present in all procedure-naive invasive breast carcinoma samples in the present study and their spatial distribution pattern, as well as abundance, differed among cases. TAMs were a predominant component near the necrotic core, in adipose tissue adjacent to the tumor and in the tumor stroma. Further investigation of TAM accumulation and phenotypic distribution at different locations may improve the understanding of their clinical implications.

E-selectin turnover is short, and it is shed into the circulation as a circulating form of E-selectin, soluble (s)E-selectin (42). sE-selectin has been used as a surrogate marker for vessel inflammation since sE-selectin expression levels appear to be associated with the vascular E-selectin present on the surface of the endothelial cells (43-48). For example, the sE-selectin expression level was significantly higher among patients with metastatic breast cancer compared with that of healthy counterparts (33.5 vs. 21.8 ng/ml; P<0.01), as well as in patients with liver metastasis compared with those without (55.3 vs. 26.0 ng/ml; P<0.0001). Thus, increased expression levels of sE-selectin were associated with reduced overall survival in breast cancer (49). Similarly, pre-surgical sE-selectin expression levels were higher in patients with colorectal cancer (43 ng/ml) compared with patients with benign diseases (43 ng vs. 31 ng/ml) and were positively associated with carcinoembryonic antigen tumor marker and poorer prognosis (both P<0.001) (50). However, a study of microarray data from 1,809 breast cancer patients with no previous treatment history revealed that E-selectin expression level was associated with longer survival times (HR, 0.67; CI 95%, 0.54-0.83; P=0.001) (51). In the present study, E-selectin expression level in breast tumor tissue samples was more abundant in females >60 years compared with those ≤60. Elevated E-selectin expression level among elderly females may be attributed to age-associated inflammation due to comorbidities, since sE-selectin expression levels are reported to be high in chronic inflammatory conditions, including arthritis (52,53), diabetes (52), atherosclerosis (54) and alcoholism (55). However, E-selectin expression level in the tumor was not associated with OS following age adjustment in the present study (data not shown). The survival implications of E-selectin expression may require integration of area-specific expression (tumor or necrosis vs. stroma), type of survival (overall vs. disease specific) and comorbidity status. In conclusion, tumor inflammation and E-selectin expression levels were identified to be positively correlated with TAMs, and the abundance of TAMs present in the tumor was an independent prognostic factor in invasive breast tumors.

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