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Recommended Citation
Morita, Yoshihiro; Zhang, Roy; Leslie, Macall; Adhikari, Smita; Hasan, Nafis; Chervoneva, Inna; Rui, Hallgeir; and Tanaka, Takemi, "Pathologic evaluation of tumor-associated macrophage density and vessel inflammation in invasive breast carcinomas" (2017). Department of Pharmacology and Experimental Therapeutics Faculty Papers. Paper 84.
https://jdc.jefferson.edu/petfp/84
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Pathologic evaluation of tumor-associated macrophage density and vessel inflammation in invasive breast carcinomas

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Received March 9, 2017; Accepted May 16, 2017

DO: 10.3892/ol.2017.6466

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-naïve 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 multivariable 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 levels. A greater prevalence of TAMs and tumor inflammation was significantly associated with shorter overall survival times. E-selectin expression levels were significantly higher in tumor vessels among elderly patients, but were not associated with 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 metastasis 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 degradation, epithelial-mesenchymal transition, metastasis, therapy response, and immunosuppression (20-22).

Macrophages originate from peripheral blood mononuclear 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

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Key words: E-selectin, cluster of differentiation 68, tumor-associated macrophages, breast cancer, inflammation, overall survival, immunohistochemistry
microenvironment (23). For successful tissue migration, circu-
lating 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 x (sLe x), cluster of differentiation (CD)44, cutaneous
lymphocyte-associated antigen and P-selectin glycoprotein
ligand-1 (29-31). E-selectin (also known as CD62E, endo-
theial 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 x - 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 anno-
tated information regarding demographics, estrogen receptor
(ER) expression, histology grade and overall survival (OS)
were used in final analyses.

Immunohistochemistry. For quality control, cases were first
immunohistochemically stained with anti-vimentin monoclona-
lar antibody (cat. no. 550513; BD Biosciences, San Jose,
CA, USA) at a 1:250 dilution overnight at 4°C. Those without
vimentin reactivity were removed from the study. Double
immunohistochemistry was performed using formalin-fixed
paraffin-embedded tumor sections (4 µm thickness). Briefly,
following deparaffinization and rehydration, antigen retrieval
was performed using Envision Flex Target Retrieval Solution
(pH 6.1; Dako; Santa Clara, CA, USA) in a pressure cooker
for 20 min at 102°C. Endogenous peroxidase and nonspecific
epitopes were blocked with 0.3% hydrogen peroxide in abso-
lute methanol for 30 min at room temperature and 5% normal
horse serum and 1% normal goat serum (Sigma-Aldrich;
St. Louis, MO, USA) for 1 h at room temperature. Sections
were incubated with mouse anti-E-selectin monoclonal antibody
at 1:100 (cat. no. MO20039; Neuromics, Inc., Minneapolis,
MN, USA) overnight at room temperature. Following washing with PBS and subsequent blocking with
5% normal horse serum and 1% normal goat serum for 5 min
at room temperature, the slides were incubated with pre-dilute
secondary horseradish peroxidase (HRP)-polymer conjugated
anti-mouse IgG (cat. no. K4001; Dako) for 30 min at room
temperature. HRP was detected using 3,3'-diaminobenzidine
(DAB; Biocare Medical LLC, Paheco, CA, USA) substrate
for 10 min at room temperature and enhanced using DAB Sparkle
(Biocare Medical LLC) for 1 min at room temperature.
Residual antibodies were eluted using Denaturing solution
(Biocare Medical LLC) at 1:3 dilution for 3 min at room
temperature to ensure no cross reaction between the first and
second staining. Slides were blocked with 5% normal horse
serum and 1% normal goat serum for 5 min at room tempera-
ture 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 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.
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

Figure 1. TAM and E-selectin expression levels in breast tumor tissues. (A-C) Single immunohistochemical staining of CD68+ TAMs for analysis of the distribution at various areas of the invasive breast carcinoma tissues (red). (D-F) Differential E-selectin expression in vessel surface in breast carcinomas (brown). The images are representative at a final magnification of x200. Scale bar indicates 100 µm. V, vessels; A, adipocytes; CD68, cluster of differentiation 68.

Figure 2. Double immunohistochemistry of TAM and E-selectin. (A and B) Spatial association of CD68+ TAM and E-selectin in the tumor stroma of invasive breast carcinoma tissues. (C) Absence of E-selectin expressing vessels in necrotic area. (D) Presence of CD68+ TAM and E-selectin in stroma of non-carcinoma area of invasive carcinoma tissue. Brown indicates E-selectin and red indicates TAM. The images are representative at a final magnification of x200. Scale bar indicates 100 µm. V, vessels; A, adipocytes; C, cancer cells; N, necrotic area; CD68, cluster of differentiation 68.
Table II. Association between CD68+ TAMs, and E-selectin expressing vessels and tumor inflammation in procedure-naïve invasive breast carcinoma tissues.

| Variables          | E-selectin | Inflammation |
|--------------------|------------|--------------|
| CD68               | 0          | -            |
|                    | 1+         | +            |
|                    | 2+         | +            |
|                    | 3+         | +            |
| 0                  | 0          | 0            |
| 1+                 | 3          | 1            |
| 2+                 | 1          | 3            |
| 3+                 | 2          | 4            |

P=0.030, r=0.302

E-selectin: 0, no immunohistochemical reaction; 1+, weakly; 2+, moderately; 3+, strongly. CD68: 0, ≤10 CD68+ cells; 1+, 11-20 CD68+ cells; 2+, 21-100 CD68+ cells; 3+, ≥100 CD68+ cells.

Discussion

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. Diagnostic needle biopsy became popular in the 1990s, and neoadjuvant chemotherapy for relatively large, locally advanced tumors emerged in the 2000s. 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. 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) and 77.6% (n=22) in frozen breast tumor tissue sections. A previous study by Charpin et al. 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. did not determine an association between E-selectin expression level 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, although it is yet to be determined which of these is more clinically important for prognosis. Unlike T-lymphocytes, whose phenotypes are classified by differentiation, the TAM phenotype is plastic and determined by its surrounding microenvironment. For example, the M1 phenotype can switch to M2 in response to T helper 2-released cytokines, including IL-13 and IL-4. Accordingly, the overall composition and
balance of immune subsets determines the pro-tumorigenic potential and fate of the tumor. TAMs were present in all procedure-naïve 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.

Acknowledgements

The present study was supported by the National Institutes of Health (grant no. 1R01CA160271-01A1). The authors would like to thank Lynsie Morris for the technical assistance provided.
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