Immunobiology 222 (2017) 31–38

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

CD68+, but not stabilin-1+ tumor associated macrophages in gaps of ductal tumor structures negatively correlate with the lymphatic metastasis in human breast cancer

Mikhail Buldakov[a,b,1], Marina Zavyalova[a,b,c,1], Nadezhda Krakhmal[a,c], Nadezhda Telegina[c], Sergei Vtorushin[a,c], Irina Mitrofanova[a], Vladimir Riabov[a,d], Shuiping Yin[d], Bin Song[d], Nadezhda Cherdyntseva[a,b], Julia Kzhyshkowska[a,d,e,*]

[a] Laboratory for Translational Cellular and Molecular Biomedicine, Tomsk State University, Pr. Lenina, 36, 634050 Tomsk, Russia
[b] Tomsk Cancer Research Institute, Per. Kooperativny, 5, 634050 Tomsk, Russia
[c] Siberian State Medical University, Moskovskiy Trakt, 2, 634050 Tomsk, Russia
[d] Department of Innate Immunity and Tolerance, Institute of Transfusion Medicine and Immunology, Medical Faculty Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany
[e] German Red Cross Blood Service Baden-Württemberg—Hessen, Friedrich-Ebert Strasse 107, 68167 Mannheim, Germany

ARTICLE INFO

Article history:
Received 2 August 2015
Received in revised form 3 September 2015
Accepted 4 September 2015
Available online 8 September 2015

Keywords:
Tumor-associated macrophages
CD68
Stabilin-1
Intratumoral heterogeneity
Lymphatic metastasis
Breast cancer

ABSTRACT

Tumor associated macrophages (TAM) support tumor growth and metastasis in several animal models of breast cancer, and TAM amount is predictive for efficient tumor growth and metastatic spread via blood circulation. However, limited information is available about intratumoral TAM heterogeneity and functional role of TAM subpopulations in tumor progression. The aim of our study was to examine correlation of TAM presence in various morphological segments of human breast cancer with clinical parameters. Thirty six female patients with nonspecific invasive breast cancer T1-4N0-3M0 were included in the study. Morphological examination was performed using Carl Zeiss Axio Lab.A1 and MiraxMidiZeiss. Immunohistochemical and immunofluorescence/confocal microcopy analysis was used to detect CD68 and stabilin-1 in 5 different tumor segments: (1) areas with soft fibrous stroma; (2) areas with coarse fibrous stroma; (3) areas of maximum stromal-and-parenchymal relationship; (4) parenchymal elements; (5) gaps of ductal tumor structures. The highest expression of CD68 was in areas with soft fibrous stroma or areas of maximum stromal-and-parenchymal relationship (79%). The lowest expression of CD68 was in areas with coarse fiber stroma (23%). Inverse correlation of tumor size and expression of CD68 in gaps of tubular tumor structures was found ($R = -0.67$; $p = 0.02$). In case of the lymph node metastases the average score of CD68 expression in ductal gaps tumor structures was lower (1.4 ± 0.5) compared to negative lymph nodes case (3.1 ± 1.0; $F = 10.9$; $p = 0.007$). Confocal microscopy identified 3 phenotypes of TAM: $CD68^+$/stabilin-1^-; $CD68^+$/stabilin-1^+ (over 50%); and $CD68^-$/stabilin-1^+. However, expression of stabilin-1 did not correlate with lymph node metastasis. We concluded, that increased amount of CD68+TAM in gaps of ductal tumor structures is protective against metastatic spread in regional lymph nodes.

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CCL, CC chemokine ligand; Cy, cyanine; ECM, extracellular matrix; EGF, epidermal growth factor; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; HRP, horseradish peroxidase; IHC, immunohistochemistry; IL, interleukin; LN, lymph node; MMP, matrix metalloproteinase; TAM, tumor associated macrophages; uPA, urokinase-type plasminogen activator; VEGF, vascular endothelial growth factor.

* Corresponding author at: Department of Innate Immunity and Tolerance, Institute of Transfusion Medicine and Immunology, Medical Faculty Mannheim, Heidelberg University, Theodor-Kutzer Ufer 1-3, D-68167 Mannheim, Germany. Fax: +49 621 383 9721.
E-mail addresses: julia.kzhyshkowska@medma.uni-heidelberg.de, julia.kzhyshkowska@gmail.com, julia.kzhyshkowska@umm.de (J. Kzhyshkowska).
1 Michael Buldakov and Marina Zavyalova contribute equally to this study.

http://dx.doi.org/10.1016/j.imbio.2015.09.011

1. Introduction

The supportive role of tumor associated macrophages (TAM) for tumor progression was primarily demonstrated in animal breast cancer models (Noy and Pollard, 2014). It was established that TAM belong to alternatively activated macrophages and can support tumor growth and metastasis using multiple mechanisms. For instance, TAM support survival and induce motility of breast cancer cells by producing epidermal growth factor (EGF) (Goswami et al., 2005). In spontaneous and ectopic models of breast cancer TAM produce pro-angiogenic factors such as VEGF-A contributing to angiogenesis and progression to malignancy, tumor cell growth and dissemination (Noy and Pollard, 2014; Gazzaniga et al., 2007; Lin et al., 2006; Sierra et al., 2008). Simultaneously with release of pro-angiogenic factors, the ability of TAM to produce matrix-degrading enzymes (MMP-2, MMP-9, uPA, cathepsins) and tumor cell-recruiting factors such as CCL-18 is associated with stimulation of breast tumor cell invasiveness and metastatic spread (Chen et al., 2011; Gocheva et al., 2010; Qian and Pollard, 2010). Moreover, TAM closely associate with blood vessels and promote tumor cells intravasation resulting in their escape into circulation and formation of distant metastases (Qian and Pollard, 2010; Pollard, 2008; Wyckoff et al., 2007).

However, in humans, not only positive but also negative correlations between TAM and various parameters of tumor growth and spread were demonstrated indicating much more complex mechanism of tumor cell-microenvironmental crass-talk. TAM density in different cancers is associated with incidence of distant metastases. TAM infiltration has positive or inverse correlation with metastases depending on tumor type. For example, high infiltration by CD68+ TAM was associated with higher risk of distant metastases in triple-negative breast cancer (Yuan et al., 2014). In addition, higher numbers of histological structures named tumor microenvironment for metastasis (TMEM) that represent close association of CD68+ macrophages, endothelial, and invasive tumor cells was associated with higher risk of distant metastasis in ER+/HER2–breast cancer (Rohan et al., 2014). In contrast, TAM infiltration was associated with suppression of metastases in high grade osteosarcoma (Buddingh et al., 2011). Similarly, higher density of CD68+ TAM was associated with lower incidence of hepatic metastases in colon cancer (Zhou et al., 2010).

In terms of lymphatic metastasis, mostly supportive role of TAM was demonstrated. For example, positive correlation between overall density of CD68+ TAM and lymph node (LN) metastases was found in human papillary thyroid carcinoma, endometrial cancer, cervical cancer, pulmonary adenocarcinoma, gastric cancer, breast cancer and others (Ding et al., 2012; Ohno et al., 2004; Qing et al., 2012; Riabov et al., 2014; Schoppmann et al., 2002; Takanami et al., 1999; Wu et al., 2012). Detrimental role of TAM in lymphatic metastases was mainly associated with their ability to produce pro-lymphangiogenic factors VEGF-C and VEGF-D that induce growth of intratumoral lymphatic vessels (Riabov et al., 2014; Iwata et al., 2007; Schoppmann et al., 2006; Song et al., 2013; Yang et al., 2011). They also release matrix-remodeling enzymes including MMP-2, MMP-9, and urokinase plasminogen activator (uPA) known to contribute to lymphangiogenesis (Riabov et al., 2014). Pro-lymphangiogenic and pro-metastatic properties of TAM were linked to their M2-like phenotype with preferential production of IL-10, CCL-18, and CCL-22 (Chen et al., 2011; Qing et al., 2012; Tsujikawa et al., 2013).

However, in the majority of studies only overall density of CD68+ TAM was analyzed and associated with LN metastases (Riabov et al., 2014). Insufficient attention was given to the intratumoral heterogeneity of TAM subtypes, in particular in relation to the different intratumoral structures. In human breast cancer following intratumoral compartments can be defined: (1) areas with soft fibrous stroma characterized by pronounced inflammatory infiltrates that are beneficial for invasive cell growth (Ham and Moon, 2013); (2) areas with coarse fibrous stroma containing collagen fibers and characterized by impaired synthesis of extracellular matrix proteins (ECM) (Campbell et al., 2011; Eiro et al., 2012; Ruffell et al., 2012; Tang, 2013); (3) areas of maximum stromal-and-parenchymal relationship revealing certain similarities with soft fibrous stroma (Mahmoud et al., 2012); (4) parenchymal elements; (5) gaps of ductal tumor structures (Pinder, 2010). All five stromal subtypes demonstrate functionally distinct areas of tumor mircoenvironment with not yet identified mechanistic role in metastatic spread.

In the present study we assessed the distribution of TAM in five distinct intratumoral morphological compartments and examined the correlation with clinical parameters of tumor progression. For the first time we demonstrated that amount of CD68+ macrophages in gaps of tubular tumor structures has negative correlation with lymph node metastasis. The highest amount of CD68+macrophages was present in the areas of soft fibrous stroma or areas of maximum stromal-and-parenchymal relationship (79%). We also identified three phenotype of TAM: $CD68^+$/stabilin-1^-; $CD68^+$/stabilin-1^+ (over 50%); and $CD68^-$/stabilin-1^+. However, expression of stabilin-1 did not correlate with lymph node metastasis. Our data suggested that increased amount of CD68 + TAM in gaps of ductal tumor structures is protective against metastatic spread in regional lymph nodes, and that these metastasis-protective TAM do not depend on pronounced M2-like activation.

2. Materials and methods

2.1. Patients

Thirty six female patients with nonspecific invasive breast cancer T1-4N0-3M0, who were treated in General Oncology Department of Tomsk Cancer Research Institute (Tomsk, Russia) from January 1999 to January 2007, were included in the present study. The study was approved by the Local Committee for Medical Ethics of our Institute (protocol No. 13 from 09.27.2014), and informed consents were obtained from all patients prior to analysis. Patients did not receive preoperative treatment. The mean age of women with breast cancer was 60.8 ± 11.3 years. Menstrual function was preserved in 7 (19%) patients, 29 women (81%) had menopause. The histological type of breast cancer was defined according to the WHO recommendations (Geneva, 2012) and corresponded to nonspecific invasive carcinoma in all cases.

2.2. Antibodies

The following primary antibodies were used: mouse monoclonal anti-human CD68 (BD Biosciences) and rabbit polyclonal anti-stabilin-1 RS1 (Kzhyshkowska et al., 2008). Super Sensitive Polymer-HRP detection system was used for immunohistochemical analysis (BioGenex, USA). Secondary antibodies for immunofluorescent staining were conjugated with Alexa488 (anti-mouse) and with Cy3 (anti-rabbit) (Dianova).

2.3. Immunohistochemical analysis

Fresh tumor tissues were fixed in 10% neutral formalin (Karbolit, Russia) for 24 h, rinsed with mixture of isopropanol (Biovitrum, Russia), and embedded in paraffin (Biovitrum, Russia). The antigen unmasking was performed using the PT Link module (Dako, Denmark) in a buffer with a high pH value. To visualize the antigen-antibody reaction, the Super Sensitive Polymer-HRP detection system was used (BioGenex, USA). Immunohistochemical analysis was performed by light microscope «Carl Zeiss Axio Lab.A1»