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Cytokines secreted by macrophages isolated from tumor microenvironment of inflammatory breast cancer patients possess chemotactic properties

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ABSTRACT

Although there is a growing literature describing the role of macrophages in breast cancer, the role of macrophages in inflammatory breast cancer (IBC) is unclear. The aim of present study was to isolate and characterize tumor associated macrophages of IBC and non-IBC patients and define their role in IBC. Tumor infiltrating monocytes/macrophages (CD14+ and CD68+) were measured by immunohistochemistry using specific monoclonal antibodies. Blood drained from axillary vein tributaries was collected during breast cancer surgery and the percentage of CD14+ in the total isolated leukocytes was assessed by flow cytometric analysis. CD14+ cells were separated from total leukocytes by immuno-magnetic beads technique and were cultured overnight. Media conditioned by CD14+ were collected and subjected to cytokine profiling using cytokine antibody array. Wound healing and invasion assays were used to test whether cytokines highly secreted by tumor drained macrophages induce motility and invasion of breast cancer cells. We found that macrophages highly infiltrate into carcinoma tissues of IBC patients. In addition blood collected from axillary tributaries of IBC patients is highly enriched with CD14+ cells as compared to blood collected from non-IBC patients. Cytokine profiling of CD14+ cells isolated from IBC patients revealed a significant increase in secretion of tumor necrosis factor-α; monocyte chemoattractant protein-1/CC-chemokine ligand 2; interleukin-8 and interleukin-10 as compared to CD14+ cells isolated from non-IBC patients. Tumor necrosis factor-α, interleukin-8 and interleukin-10 significantly increased motility and invasion of IBC cells in vitro. In conclusion, macrophages isolated from the tumor microenvironment of IBC patients secrete chemotactic cytokines that may augment dissemination and metastasis of IBC carcinoma cells.

1. Introduction

Inflammatory breast cancer (IBC) is the most aggressive and lethal form of breast cancer. Women often present with IBC at a young age (Nouh et al., 2011), are more likely to have metastatic disease at the time of diagnosis (Wedam et al., 2006) and have a shorter survival as compared to women with non-IBC (Chang et al., 1998). IBC is characterized by invasion into dermal lymphatic vessels where IBC cells form tumor emboli (Van Laere et al., 2006). Spreading of tumor emboli within lymphatic and blood vessels leads to distant metastasis and multi-organ failure in IBC patients (Tsoi et al., 2010). Dissemination of carcinoma cells can be regulated by cues from the inflammatory cells within the tumor microenvironment. Macrophages, which are the major inflammatory cells that infiltrate into breast tumors (Mukhtar et al., 2011; Pollard, 2008), contribute to high levels of growth factors, hormones, and

Abbreviations: CCL-2, CC-chemokines ligand 2; CCR2, C-C chemokine receptor type 2; c-Fms, macrophage colony stimulating factor-1 receptor; HCMV, human cytomegalovirus; IBC, inflammatory breast cancer; IL-4, interleukin-4; IL-8, interleukin- 8; IL-10, interleukin-10; IL-13, interleukin-13; INF-γ, interferon-γ; M1, classical activated macrophages; M2, alternatively activated macrophages; MCP-1, monocyte chemoattractant protein-1; MMP-2, matrix metalloproteinases-2; MMP-9, matrix metalloproteinases-9; NF-κB, Nuclear factor kappa beta; PI3K, phosphatidylinositol-3 kinas; STAT3, signal transducer and activator of transcription; TAMs, tumor associated macrophages; TNF-α, tumor necrosis factor-alpha.

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cytokines (Aaltomaa et al., 1992; Georgiannos et al., 2003) and are designated as tumor associated macrophages (TAMs). Within the tumor microenvironment macrophages polarize into heterogeneous subpopulations depending on the type of external stimuli they receive (Cassetta et al., 2011). Among TAM subpopulations are: (1) 'classical activated macrophages' (M1), which are activated by pro-inflammatory agents such as interferon-γ (INF-γ) and tumor necrosis factor-α (TNF-α) (Cassetta et al., 2011); (2) 'alternatively activated macrophages'(M2) developed in response to IL-4 and IL-13 (Gordon and Martinez, 2010; Mantovani and Sica, 2010); and 3) 'regulatory macrophages' that express anti-inflammatory cytokines and increase tumor growth, invasion and metastasis (Mosser and Edwards, 2008). Classical activation induces the secretion of pro-inflammatory mediators by the macrophages and recruitment of T-cells as in an early inflammatory response (Ojalvo et al., 2009). M2 macrophages exhibit anti-parasite, immunosuppressive, wound healing and tissue remodeling properties (Gordon, 2003; Martinez et al., 2009). Indeed TAMs can regulate multiple mechanisms associated with dissemination of carcinoma cells. For instance, TAMs secrete proteolytic enzymes such as matrix metalloproteinases-2 and 9 (MMP-2 and MMP-9) that can degrade components of the basement membrane, thereby facilitating tumor cell intravasation and spreading in blood and lymphatic vessels (Hagemann et al., 2005; Mantovani et al., 2006). Increases in expression of MMPs and their inhibitors in TAMs have been found to correlate with distant metastasis of invasive ductal carcinomas (Gonzalez et al., 2007). The cysteine protease cathepsin B (Ibrahim et al., 2006) is expressed by TAMs in a transgenic mouse model for mammary carcinoma (Vasiljeva et al., 2006) and co-expressed with interleukin-10 (IL-10) in late stage lung cancer (Daurkin et al., 2011). We have previously shown that high levels of cathepsin B within the IBC microenvironment are associated with lymphatic metastasis (Nouh et al., 2011). Furthermore, TAMs secrete cytokines that control physiological mechanisms associated with tumor progression, i.e.,interleukin-8 (IL-8),which induces angiogenesis; tumor necrosis factor-alpha (TNF-α), which stimulates tumor growth and invasion (Dirkx et al., 2006), as well as immunosuppressive cytokines, i.e., monocyte chemoattractant protein-1 (MCP-1) or CC-chemokine ligand 2 (CCL-2) and IL-10(Daurkin et al., 2011). In fact TAMs play crucial roles in the dissemination of breast cancer cells. This is evident from intravital imaging which has provided new insights into how subpopulations of TAMs patrol inside blood vessels in the tumor microenvironment and at the tumor margins (Egeblad et al., 2008). Thus macrophages are considered to be potential diagnostic and therapeutic targets (Mukhtar et al., 2011). Therapeutic strategies include targeting macrophage recruitment to the tumor site by CCL-2 neutralizing antibodies (Balkwill and Mantovani, 2010); or altering macrophage development by targeting macrophage colony stimulating factor-1 receptor (c-Fms) using the tyrosine kinase inhibitor imatinib (Dewar et al., 2005).

Although macrophages have been identified as major cellular components of the non-IBC microenvironment their role in IBC is not well understood (Kleer et al., 2000). Herein, we show that IBC is characterized by high infiltration and venous drainage of macrophages that secrete cytokines different from those secreted by macrophages from non-IBC patients. Moreover, we identified major cytokines that may contribute to IBC invasion and motility and can be therapeutically targeted.

2. Materials and methods

2.1. Patients

For the purpose of patient enrollment in this study, we obtained Institutional Review Board (IRB) approval from the ethics committee of Ain-Shams University, Cairo, Egypt and the National Cancer Institute (NCI), Cairo University, Giza, Egypt. Patients were enrolled from outpatient breast clinics of Ain Shams University hospitals and NCI Cairo University during the period of January 2010–January 2012. All patients signed consent form including approval for publication of the study results before participation. Inclusion criteria of breast cancer patients were dependent upon a combination of clinical, mammographic, ultrasound and pathological diagnoses as we described before (Nouh et al., 2011). Clinical diagnosis of IBC was applied, according to the American Joint Committee on Cancer (AJCC) T4d designation for IBC (for review see (Dawood et al., 2010), i.e., when a patient presented with a diffuse erythema, peau d'orange and edema of the breast. For IBC patients, pathological confirmation of the clinical diagnosis was dependent upon examination of both skin and core biopsies. Non-IBC patients of stage II–III were also included as a comparison group. Based on the criteria described here, we enrolled 66 patients; 39 were diagnosed as non-IBC and 27 as IBC.

2.2. Localization of tumor associated macrophages in breast tumor microenvironment

Cancer tissues excised from modified radical mastectomies were divided into 2 halves: one fixed in 10% neutral buffered formalin and processed into paraffin blocks for routine diagnosis and immunohistochemistry (IHC) and one snap frozen in liquid nitrogen for molecular and biochemical studies. Pathological data [tumor size, tumor grade (Genestie et al., 1998), presence of lymphovascular invasion, and tumor emboli (Bonnier et al., 1995; Gong, 2008) were assessed, reviewed and tabulated for statistical analysis. For IHC staining of macrophages within paraffin embedded tissue, 5 μm sections were first deparaffinized and rehydrated followed by antigen retrieval. Tissue sections were incubated for 1 h at room temperature with the primary antibodies: monoclonal mouse anti-human CD14 (1:50) antibody (CBL453) from Chemicon (Temecula, CA, USA) and monoclonal mouse anti-human CD68 (1:50) antibody (M0814) from (DakoCytomation, Denmark). Immunostaining for each marker was achieved as we described before (Al-Raawi et al., 2011; Nouh et al., 2011) using EnVision+ Dual Link System-HRP (DAB+) from (DakoCytomation, Denmark). Negative control slides were run in parallel in which the primary antibodies were replaced with PBS. Nuclei were counterstained with hematoxylin and specimens were rinsed in phosphate buffered saline (PBS) and mounted using Permount® for microscopic examination. Two independent readers (M.A.N. and M.M.M.) assessed immunostaining for CD14+ and CD68+ using light microscopy (Olympus, CX41, Japan). Discordant results were resolved by consultation with a third reader (H.I.). Scoring of immunostaining was done by counting cells positive for CD14 and CD68 (cytoplasmic and membranous staining) in paraffin embedded carcinoma tissues of non-IBC ($n = 39$) and IBC ($n = 27$) patients: "0", no immunostaining was observed; "+", less than 10% of cells showed positive staining; "++", 10–50% cells showed positive staining; and "+++", more than 50% cells showed positive staining (Nouh et al., 2011).

2.3. Blood sample collection and isolation of tumor associated monocytes/macrophages

During modified radical mastectomy, 15–20 ml blood that had drained from the tumor microenvironment through axillary tributaries was collected by the surgeon in heparinized tubes. Collected blood was transferred directly to the laboratory for isolation of leukocytes as we have described (El-Shinawi et al., 2010). Briefly, blood was diluted with an equal amount of PBS, pH 7.2, at room temperature. Mononuclear cells were separated by