NEOPLASIA
www.neoplasia.com

Volume 16 Number 7 July 2014 pp. 586–594 586

Tumor Interstitial Fluid Pressure—A Link between Tumor Hypoxia, Microvascular Density, and Lymph Node Metastasis

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Abstract

High microvascular density (MVD) in the primary tumor has been shown to be associated with increased incidence of lymph node metastases and poor clinical outcome. Other investigations have revealed that a large fraction of hypoxic tissue in the primary tumor is associated with metastatic disease and impaired survival. These data are apparently incompatible because tumor hypoxia is primarily a consequence of poor oxygen supply caused by an inadequate vasculature with increased intervessel distances. Here, we provide an explanation of these observations. Human melanoma xenografts were used as preclinical cancer models. Tumors that metastasized to lymph nodes showed higher interstitial fluid pressure (IFP) than those that did not metastasize, and compared with tumors with low IFP, tumors with high IFP showed large hypoxic fractions centrally, high MVD in the periphery, high peritumoral density of lymphatics, and elevated expression of vascular endothelial growth factor A (VEGF-A) and VEGF-C. Significant correlations were found between peripheral MVD and central hypoxia, and lymph node metastasis was associated with high values of both parameters. These findings suggest that the outcome of cancer may be associated with both high MVD and extensive hypoxia in the primary tumor. We propose that proangiogenic factors are upregulated in the tumor center and that the outward interstitial fluid flow caused by the elevated IFP transports these factors to the tumor surface where they evoke hemangiogenesis and lymphangiogenesis, and consequently, that the IFP serves as a link between tumor hypoxia, peripheral tumor hemangiogenesis, peritumoral lymphangiogenesis, and lymph node metastasis.

Neoplasia (2014) 16, 586–594

Introduction

Angiogenesis plays an important role in the progression of malignant diseases [1], and there is substantial evidence that the prognosis of patients with cancer is associated with the angiogenic potential of the primary tumor [2–4]. Clinical studies involving a wide range of cancer types have demonstrated significant correlations between disease-free or overall survival rate and tumor microvascular density (MVD) [4]. Furthermore, tumor MVD has been shown to be significantly higher in patients with metastases than in metastasis-free patients in many cancer diseases, including breast carcinoma [5], prostate carcinoma [6], squamous cell carcinoma of the head and neck [7], lung carcinoma [8], bladder carcinoma [9], ovarian carcinoma [10], and malignant melanoma [11–13]. Interestingly, most of these cancer types metastasize to regional lymph nodes through functional lymphatics adjacent to the primary tumor, whereas MVD was scored by determining the density of blood vessels in vascular hot spots within the primary tumor [4]. Vascular hot spots can occur anywhere in tumors but are seen primarily in the invasive front [14], consistent with the observation that MVD increases gradually from the center to the periphery of tumors [15].

Abbreviations: HF, hypoxic fraction; IFP, interstitial fluid pressure; LVD, lymph vessel density; LYVE-1, lymphatic endothelial hyaluronan receptor-1; MVD, microvascular density; VEGF, vascular endothelial growth factor

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Financial support was received from the Norwegian Cancer Society and the South-Eastern Norway Regional Health Authority. Conflicts of interest: The authors have no conflicts of interest to disclose.

Received 12 June 2014; Revised 8 July 2014; Accepted 11 July 2014

1476-5586/14
http://dx.doi.org/10.1016/j.neo.2014.07.003

The microvascular networks of most tumors have significant morphologic and architectural abnormalities [16–18]. These abnormalities result in elevated geometric and viscous resistance to blood flow, increased transvascular fluid flow, inadequate perfusion, and heterogeneous supply of oxygen and nutrients [16,17]. Therefore, many macroscopic tumors show a physiological microenvironment characterized by nutrient deprivation, high interstitial fluid pressure (IFP), and hypoxia [17,18]. There is significant evidence that these abnormalities promote metastatic dissemination and tumor growth at regional and distant sites [18,19]. Several preclinical studies have suggested that tumors with high fractions of hypoxic cells metastasize more frequently than genetically equivalent tumors with low hypoxic fractions (HFs) [20–24], and highly elevated IFP has been shown to be associated with increased incidence of pulmonary and lymph node metastases in human melanoma and cervical carcinoma xenografts [25,26]. Clinical investigations have revealed that extensive hypoxia in the primary tumor is associated with malignant progression, development of metastatic disease, and poor disease-free and overall survival rates in a large number of cancer types [27–30]. Studies of patients with locally advanced cervical carcinoma have suggested that high IFP in the primary tumor is linked to high incidence of distant metastases, pelvic recurrence after radiation therapy, and impaired survival [31,32].

Large HFs in tumors and hypoxia-induced resistance to radiation therapy have been shown to be associated with low tumor MVD in several cancer types, including cervical carcinoma [33] and carcinoma of the head and neck [34], consistent with the generally accepted view that tumor hypoxia is primarily a consequence of poor oxygen supply caused by elevated resistance to blood flow and increased intervessel distances [17,18]. The significant number of studies suggesting that lymph node metastasis and poor survival rates are associated with high tumor MVD is apparently inconsistent with those suggesting that metastasis and poor outcome are a consequence of tumor hypoxia. It has been proposed, however, that high MVD may result from hypoxia-induced angiogenesis mediated by proangiogenic factors that are upregulated by hypoxia and, hence, that the two sets of apparently inconsistent observations are not mutually exclusive. Although hypoxia-induced angiogenesis may be important, this is not a satisfactory explanation because hypoxic tissue usually exists in the central regions of tumors [17], whereas high MVD and microvascular hot spots are seen primarily in the tumor periphery [14].

In the work reported in this communication, possible relationships between tumor hypoxia, intratumoral MVD, and lymph node metastasis were studied by using human melanoma xenografts as experimental models of human cancer. The main purpose of the study was to provide plausible explanations for 1) the observations that cancer metastasis may be associated with both the fraction of hypoxic tissue and the MVD of the primary tumor and 2) the observations that the density of blood vessels within primary tumors may be associated with lymphogenous metastatic spread. We hypothesized that the elevated IFP and the associated interstitial fluid flow of tumors may serve as a link between tumor hypoxia, MVD, and lymph node metastasis. This hypothesis was investigated by measuring the central IFP, the central fraction of hypoxic tissue, and the peripheral MVD of metastatic and non-metastatic R-18 and T-22 tumors and relating the observations to established correlations between IFP, rate of interstitial fluid flow, and lymph node metastasis.

Materials and Methods

Mice

Adult (8-10 weeks of age) female BALB/c *nu/nu* mice, bred and maintained under specific pathogen-free conditions, were used as host animals for tumors. The animal experiments were approved by the Institutional Committee on Research Animal Care and were done according to the US Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Tumors

The R-18 and T-22 human melanoma cell lines were established in our laboratory as described earlier [35]. The cells used in the present experiments were obtained from our frozen stock and were maintained in monolayer culture in Roswell Park Memorial Institute 1640 (25 mmol/l Hepes and L-glutamine) medium supplemented with 13% bovine calf serum, 250 mg/l penicillin, and 50 mg/l streptomycin. Xenografted tumors were initiated by inoculating aliquots of ~ 3.5×10^5 R-18 cells or ~ 1.0×10^6 T-22 cells intradermally into the left mouse flank. The tumors were included in experiments when they had grown to a volume of ~ 400 mm^3. R-18 and T-22 tumors of this size do not show significant regions with necrotic tissue.

Hypoxia, Density of Blood and Lymph Vessels, and Expression of Vascular Endothelial Growth Factor A and Vascular Endothelial Growth Factor C

Hypoxic tissue, blood vessels, lymphatics, vascular endothelial growth factor A (VEGF-A), and VEGF-C were detected by immunohistochemistry [36]. Pimonidazole [1-[(2-hydroxy-3-piperidinyl)-propyl]-2-nitroimidazole], injected as described previously [23], was used as a marker of tumor hypoxia, and CD31 and lymphatic endothelial hyaluronan receptor-1 (LYVE-1) were used as markers of blood and lymph vessel endothelial cells, respectively. An anti-pimonidazole rabbit polyclonal antibody (Professor Raleigh, University of North Carolina, Chapel Hill, NC), an anti-mouse CD31 rat monoclonal antibody (Research Diagnostics, Flanders, NJ), an anti-mouse LYVE-1 rabbit polyclonal antibody (Abcam, Cambridge, United Kingdom), an anti-human VEGF-A rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or an anti-human VEGF-C goat polyclonal antibody (Abcam) was used as primary antibody. Quantitative studies were carried out on preparations cut sagittally through the central regions of tumors and the surrounding skin, and four sections were analyzed for each tumor. Microvessels were defined and scored manually as described by Weidner [14]. Blood vessel density in the invasive front (peripheral MVD) was determined by counting vessels located within a 1-mm-thick band in the tumor periphery (Figure 1, *A* and *B*). Peritumoral density of lymphatics [peritumoral lymph vessel density (LVD)] was assessed by counting vessels in the surrounding skin located within a distance of 0.5 mm from the tumor surface. Fraction of hypoxic tissue was assessed by image analysis and was defined as the area fraction of the tissue showing positive pimonidazole staining. HF in the tumor center (central HF_{Pim}) was measured by analyzing the tissue located inside the invasive front, that is, further in from the tumor surface than 1 mm (Figure 1, *A* and *B*).

VEGF-A Concentration

VEGF-A concentrations were measured in tumors frozen in liquid nitrogen immediately after resection [37]. The frozen tissue was pulverized and lysed in radioimmunoprecipitation assay buffer. The samples were centrifuged and the supernatants were stored at −80°C until analysis. Total protein concentrations were determined by using