Pathology – Research and Practice 206 (2010) 753–759

Contents lists available at ScienceDirect

Pathology – Research and Practice

journal homepage: www.elsevier.de/prp

Original Article

Biological impact of vascular endothelial growth factor on vessel density and survival in multiple myeloma and plasmacytoma

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ARTICLE INFO

Article history:
Received 25 May 2010
Accepted 2 July 2010

Keywords:
iNOS
Myeloma
Plasmacytoma
VEGF
Vessel density

ABSTRACT

We compared the differences in a number of angiogenesis-related immunohistochemical parameters, including microvascular density (MVD) and tumor cell activity, between multiple myeloma (MM) and solitary plasmacytoma (SP).

Tissue sections from tumors of MM and SP were immunohistochemically stained and analyzed using ImageJ image analysis software for the expression of vascular endothelial growth factor (VEGF), VEGF receptors (Flt-1 and Flk-1), inducible nitric oxide (iNOS), and anti-apoptotic (Bcl-2) protein. Tumor tissues were cytologically graded as high-, intermediate-, or low-grade. Two pathologists determined the MVD of each section independently by recording the average number of CD34+ blood vessels in 500 unit fields. The arithmetic means for MVD were statistically analyzed using the Student's *t*-test and the significance level was calculated at *P*-value <0.001.

The results indicate a direct correlation between upregulation of iNOS/VEGF in high-grade tumors. For MM, an increase in MVD is also correlated with a high-grade. Tumor survival signaling by Bcl-2 in both SP and MM emphasizes the fact that VEGF has a bimodal role that is mainly angiogenic in MM and tumorigenic, promoting tumor cell survival in SP.

Introduction

Recent studies have emphasized the importance of neovascularization as an indispensible prerequisite for the development and metastasis of many solid tumors, including kidney, prostate, breast, and hematologic malignancies [14,30].

A clear correlation has been determined between the levels of angiogenic growth factors and the prognosis for malignant neoplasms [24,26]. Solitary plasmacytomas (SP) are solitary clonal proliferations of malignant plasma cells that are cytologically and immunophenotypically identical to plasma cell myelomas, and may occur as either extramedullary plasmacytomas (EPs) or intramedullary plasmacytomas (IPs) [8]. Multiple myelomas occur primarily in the bone marrow and usually at multiple sites [5]. Both EPs and IPs are localized forms of plasma cell dyscrasia, but IPs have a greater tendency to progress into MM [6,33]; the greater number of IP cases may conceal an occult MM at diagnosis. Among hematologic malignancies, MM is the only neoplasm that has shown a correlation between microvessel density (MVD) and prognosis [4,29,31,32].

Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic growth factors known. Among angiogenesis factors, it is characterized by its dual angiogenic and tumorigenic roles that affect the growth and spread of malignant neoplasms [19,20]. Previous studies demonstrated VEGF as an important regulator of cellular growth, survival, and migration in tumor cells [9], including stromal osteoclasts [1] and endothelial cells [3,29]. In studies of malignant plasma cell neoplasms [15,16], higher VEGF expression in tumor cells was correlated to an increase in the MVD of tumor tissues [13]. Considerable research has demonstrated the relationship between VEGF activity and tumor grade and prognosis in MM [27]. However, few reports have investigated solitary plasmacytoma (SP). Our goal was to demonstrate differences in the expression of VEGF in MM and SP in regard to its influence on tumor microvessel density and tumor cell survival.

Materials and methods

Materials

This study was conducted in accordance with the Medical Ethics Regulations at Tanta and Dammam Universities, which are consistent with the Declaration of Helsinki. A total of 50 surgical archival cases of SP and a similar number of cases of MM were

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doi:10.1016/j.prp.2010.07.006

Table 1
List of primary antibodies used in immunohistochemistry for solitary plasmacytoma and multiple myeloma.

Antibody against	Immunogen		Clone	Dilution	Distributors
	Species	Origin			
VEGF	Mouse	Culture supernatant for full length VEGF189	C1	1:50	Santa Cruz
Flk-1	Rabbit	1158–1345 amino acids of mouse Flk-1	NA	1:50	Thermo
Flt-1	Rabbit	1164–1174 amino acids of human VEGFR-1	NA	1:1000	Thermo
CD34	Human	Tonsil	QBEnd/10	1:100	Thermo
Bcl-2	Mouse	41–54 Bcl-2 peptide aminoacid sequence	C124	1:100	Dako
iNOS	Mouse	Mouse ascites fluid	NOS-IN	1:1000	Sigma
CD138	Mouse	Culture supernatant	MI15	1:50	Dako
CD20	Mouse	Daudi cell line B9E9	[a]	1:100	Chemicon
Kappa	Rabbit	A pool of human sera	NA	1:200	Dako
Lambda	Rabbit	Human urine Bence Jones protein	N10/2	1:200	Dako
IgG	Mouse	Ascites	IT28	1:100	Sigma
AgA	Mouse	EGF-stimulated A431 cell lysate	C10-3	1:100	B D Biosciences

NA, not applicable.
[a] Mouse monoclonal antibody, clone name not available.

retrieved from the files of the Department of Oral Pathology at Tanta College of Dentistry, Egypt, and from the Department of Pathology at the Medical College at Dammam University, Saudi Arabia. Cases diagnosed during the period 1990–2009 were reviewed, and 10 histopathologically representative samples of SP and a similar number of MM cases were selected. Cases were grouped into SP and MM regardless of their location (osseous or extraosseous). Selected neoplasms were cytologically subgraded according to Bartl [2] into low-grade Marschalko (characterized by predominantly small cells), intermediate-grade (with predominantly cleaved, polymorphous, asynchronous cells), or high-grade (where most tumor cells are plasmablastic).

The 20 biopsies included in this study were from the following sites: bone marrow ($n = 11$), spine ($n = 4$), maxilla ($n = 4$), skeletal muscles ($n = 3$), mandible ($n = 3$), anterior chest wall ($n = 2$), and orbit ($n = 1$). Intramedullary lesions were radiographically presented as punched-out radiolucencies, and the extramedullary lesions appeared as diffuse lumps.

Selected archived specimens were serially sectioned at a thickness of 5 μm. One set of sections was histochemically stained with hematoxylin and eosin (HE), and methyl green-pyronin (MG-P), and other sets were immunohistochemically stained with plasma cell diagnostic markers against D138, CD20, kappa and lambda light chains, IgG, IgA, markers against VEGF and its two main receptors (Flt-1 and Flk-1), anti-apoptotic Bcl-2 protein, vessel-related molecule CD34, and the angiogenesis-related enzyme iNOS.

Antibodies

The following reagents were purchased from the indicated manufacturers: rabbit polyclonal antibodies against anti-kappa and anti-lambda light chains from Dako Ltd. (Glostrup, Denmark, diluted at 1:200); mouse monoclonal antibodies against Bcl-2 and CD138 (MI15) from Dako Ltd. (1:100, 1:50, respectively); mouse monoclonal antibodies against IgG (IT28) and iNOS, from Sigma Chemicals Co. (St. Louis, MO, USA, 1:100, 1:1000, respectively); mouse monoclonal antibodies against VEGF (C-1) from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA, 1:50); CD34 (qbend/10) from Thermo Fisher Scientific Inc. (Fremont, CA, USA, 1:100); rabbit polyclonal antibodies against Flk-1and Flt-1 from Thermo (1:50, 1:1000, respectively); and mouse monoclonal antibodies against human IgA (C10-3) from BD Biosciences (San Jose, CA, USA, 1:50); and mouse monoclonal antibodies against CD20 from Chemicon International Inc. (Temecula, CA, USA, 1:100). The details for primary antibodies used in this study are summarized in Table 1.

Immunohistochemistry

Immunohistochemical experiments were performed using the Envision peroxidase system. After deparaffinization, sections were treated in 0.3% H_2O_2 in methanol for 30 min at room temperature to block endogenous peroxidase activity. The sections were then rinsed in 0.01 M phosphate buffered saline (PBS). To restore antigenicity, sections were pretreated by autoclaving in citrate buffer (pH 6.0) at 121 °C for 10 min. After antigen retrieval treatments, the sections were incubated in 5% skim milk in PBS containing 0.05% Triton X-100 (PBST) for 30 min at 37 °C to block non-specific protein binding, and the sections were then incubated at 4 °C overnight with the primary antibodies. After rinsing in PBST, the sections were incubated with goat antibodies against rabbit or mouse IgGs conjugated with peroxidase-labeled dextran polymers (EnVision+ peroxidase, rabbit/mouse, Dako) for 30 min at room temperature. The peroxidase reaction products were visualized by incubation with 0.02% 3,3′-diaminobenzidine (Dohjin Laboratories, Kumamoto, Japan) in 0.05 M Tris–HCl solution (pH 7.4) containing 0.005% H_2O_2. The sections were then counterstained with hematoxylin.

Microvessel density and statistical analysis

Because endothelial cells are particularly active in highly vascularized regions, we performed double-blind counting for microvessels in hot spots of anti-CD34 stained biopsies, as described elsewhere [22]. Briefly, each slide was scanned at 100× magnification. The five areas with the maximum number of CD34-marked microvessels were chosen as "hot spots". The microvessels in these areas were counted at 200× high-power field (hpf) using the micrometer in 500 unit squares ($1/mm^2$) of tumor tissue, and the MVD was expressed as the average number of vessels per hot spot. Then, the arithmetic means for SP and MM were analyzed by Student's *t*-test using SPSS-10 statistical software. Statistical analysis for the arithmetic mean of MVD in high-, intermediate-, and low-grade plasma cell neoplasms was also evaluated. A *P*-value of less than 0.001 was considered statistically significant. In addition, we used Tukey HSD test comparing the average (+) staining count of VEGF, iNOS, and Bcl-2 in both SP and MM.

Assessment of the staining intensity and number of positive cells

A section was considered either positive or negative according to the presence or absence of brown staining in the cytoplasm or cell membrane of tumor cells.

Similar to our procedure for vessel counting, two independent pathologists counted the number of positive cells in (+) cases. Three