Original Article

Assessment of bone marrow angiogenesis using F VIII-related antigen and its relationship to proliferating cell nuclear antigen (PCNA) in multiple myeloma.

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Fac Med Baghdad 2011; Vol. 53, No. 2
Received Sept. 2010
Accepted Feb. 2011

Summary:

Background: Multiple myeloma (MM) is a malignant clonal expansion of plasma cells. Previous studies had demonstrated that both bone marrow angiogenesis as measured by microvessel density (MVD) and PCNA were increased in variety of malignant disorders, including multiple myeloma.

Objective: Assessment of angiogenesis in bone marrow of MM patients by using immunohistochemical stain to measure microvessel density (MVD) using factor VIII –related antigen and to evaluate the expression of PCNA antigen in plasma cells of MM bone marrow sections . Also to find the correlation between these two parameters and between them and the percentage of plasma cells infiltration in bone marrow of MM.

Patients, materials and methods: This retrospective study was conducted from May 2007 to Jaunary 2008 on 37 bone marrow biopsies diagnosed as multiple myeloma, along with 10 age matched control subjects who had reactive plasmacytosis of less than 10% in their bone marrow. The cases were retrieved from recording archive files of Department of Pathology in the Teaching Laboratory of Medical City Hospital, laboratory of Al- Yarmouk hospital and AL-Yarmouk National Center of Haematology. Three sections were taken from each formalin fixed paraffin embedded bone marrow trephine biopsy of MM and of control cases. One representative section was stained with Hematoxylin and Eosin (H&E), while the other two sections were stained immunohistochemically for factor VIII-related antigen as an endothelial cell marker, and for PCNA as an indicator of the proliferative state of myeloma cells . All stained sections were examined by light microscopy and the mean vessel density (MVD) was estimated and was used for statistical analysis. For immunostained PCNA cells, the labeling index scoring system of Alves et al was adopted for estimating the percentage of positive nuclear staining.

Results: This study revealed that there was a significant increase in bone marrow angiogenesis and in the expression of PCNA in plasma cells of patients with multiple myeloma compared to control cases. Moreover this study showed that PCNA correlated significantly with bone marrow microvessel density and both parameters correlated significantly with the percent of plasma cell in the bone marrow of patient with multiple myeloma.

Conclusion : Both PCNA and angiogenesis as expressed by MVD were increased in multiple myeloma and both of them correlated with the percentage of plasma cell infiltration which reflected the activity of the disease .Moreover the proliferative activity of plasma cell as represented by PCNA expression was closely related to angiogenic activity , thus it can be proposed that both markers reflect the disease activity and they may provide additional information when included in the initial evaluation of myeloma bone marrow biopsies .

Keyword: angiogenesis; PCNA: multiple myeloma.

Introduction:

Multiple myeloma (MM) is a neoplastic plasma cell dyscrasia (PCD) characterized by a clinical pentad: (a) anemia, (b) a monoclonal protein in the serum or urine or both, (c) abnormal bone radiographs and bone pain, (d) hypercalcemia, and (e) renal insufficiency or failure .(1) Under normal conditions, the human bone marrow is supplied by a small number of blood vessels. The concentration of these vessels, or bone marrow microvessel density (MVD), is increased in various malignant disorders including multiple myeloma .(2) Angiogenesis, is the formation of new blood vessels from pre existing vascular structures .(3) It is mediated by angiogenic molecules released by tumor cells themselves and by accessory host cells such as macrophages, mast cells, and lymphocytes. In turn, the newly formed endothelial cells of the tumor can stimulate tumor growth in a paracrine fashion.(4,5) Multiple myeloma was the first hematological malignancy in which a prognostic relevance of bone marrow micro vessel density (MVD) was shown.(4,6) Recently , many studies found that soluble angiogenic factors were elevated in bone marrow and in peripheral blood sample from myeloma patients and correlations were found between disease stages , prognosis and serum levels of these angiogenic factors particularly, the basic fibroblast growth factor (bFGF) , hepatocyte growth factor (HGF) and vascular

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endothelial growth factor (VEGF), which give strong evidence for an increased angiogenic activity in bone marrow microenvironment .(4 ,7) Proliferating cell nuclear antigen (PCNA) is a homotrimeric ring-shaped protein that encircles the DNA and acts as a stationary loading platform for multiple , transiently interacting partners participating in various DNA transaction . Within the eukaryotic cell , PCNA serves as a processivity factor for DNA polymerase δ, and plays a key role in DNA replication ,repair , cell cycle regulation , and post – replicative transactions like DNA methylation and chromatin remodeling .Thus it can serve as a cell proliferative marker.(8,9,) In human PCNA gene is located on chromosome 20 .(10) It maybe expressed in normal cells and in many malignant and non malignant diseases . In normal bone marrow, PCNA normaly expressed by erythroid precursors; hematopoietic progenitor cells but not in mature neutrophils, eosinophils or erythroid cells . It is also expressed in the proliferative compartments of normal tissues such as in the intestine and the cervical squamous epithelia .(11) Abnormally high expression of PCNA was observed in many malignant diseases such as malignant lymphoma , multiple myeloma and osteosarcoma , as well as in non malignant diseases such as psoriasis , hepatic injury and endometrial proliferative disorders. (10,12,13) In Multiple myeloma high expression of PCNA correlates with many proliferative markers such as IL-10 , IL -15 (14), IL -6(15) and the nucleolar organizer regions (AgNORs) (16)as well as with the expression of the anti-apoptotic protein Bcl-xL . (17)

Aim of the study : Assessment of angiogenesis in bone marrow of patients with MM by using immunohistochemical stain to measure microvessel density (MVD) using factor VIII –related antigen / von Willebrand's factor (FVIII-vWF) and to evaluate the expression of PCNA antigen in plasma cells of MM bone marrow sections . Also to find the correlation between these two parameters and between them and the percentage of plasma cells infiltrating the bone marrow of MM.

Patients, material and methods:

This retrospective study was conducted from may 2007 to Jaunary 2008 on 37 patients whose bone marrow biopsies were diagnosed as having multiple myeloma, along with 10 age and sex matched control subjects who had reactive plasmacytosis of less than 10% in their bone marrow . The cases were retrieved from recording archive files of Department of Pathology in the Teaching Laboratory of Medical City Hospital, laboratory of Al- Yarmouk hospital and AL-Yarmouk National Center of Haematology . The examined bone marrow biopsies were obtained before starting treatment, and the patients were selected randomly regarding age and sex .Three sections were taken from each formalin fixed paraffin embedded bone marrow of MM and of control cases. One representative section was stained with Hematoxylin and Eosin (H&E), while the other two were stained immunohistochemically for factor VIII-related antigen (von Willebrand Factor) as an endothelial cell marker , and for PCNA as an indicator of the proliferative state of myeloma cells . The immunohistochemical staining used for both FVIII –related antigen and PCNA was performed with horseradish peroxidase (HRP)-labelled –streptavidin –biotin method .(18) This technique basically uses an unlabeled primary antibody, which was mouse monoclonal antibody purchased from DAKO (code no. of the kits were QBEND10 for FVIII –related antigen and PC 10 for PCNA) , it binds to its corresponding antigen, followed by a biotinylated secondary antibody to which the avidin-biotin complex (one avidin molecule, three biotin-labeled peroxidase molecules) attaches. If the sought-after antigen is present in the section, this procedure results in an antibody-antigen interaction and an enzymatic reaction that can be detected by the chromogen, diaminobenzidine (DAB), and can be visualized by light microscopy .(18) Negative controls were obtained by replacing the primary antibody with buffer saline and positive controls for each antibody were included with the samples using inflamed tonsil for FVIII-related antigen and Non Hodgkin lymphoma tissue for PCNA. For MVD estimation , each of the studied slide was first scanned at 100 x magnification ,and 5 areas with abundant microvessels were chosen and defined as " hot spot" ,the number of microvessels in each of these hot spots was determined at 400 x magnification,and the number was divided by HPF area of that field (HPF using Olympus microscope at 400x is 0.1885 mm2) yielding MVC/Area or the mean vessel density (MVD) (19) .During the counting process, large vessels, tortuous vessels, and vessels in the periosteum or bone and open sinusoids were excluded. Area of staining with no discrete breaks were counted as single vessels, and the presence of a lumen was not require. Megakaryocytes which stain with anti –vWF will be easily identified due to its characteristic morphology and size. Regarding PCNA expression , it was evaluated semi-quantitatively according to the scoring system adopted by Alves et al (20), by counting in each sample the number of positive nuclear staining at 400X magnification , regardless of the intensity of the stain , in 1000 tumor cells from five different randomly selected representative fields. Lableing index for each field was calculated which in equal to the (number of positive cells/number of total cells) x100 .The mean value of labeling indices of the five fields was considered to be the label index for the case. According to the labeling index the cases were grouped into four scores, as shown in table 1.

Table 1: Scoring of immunohistochemical results of PCNA as adopted by Alves et al. ([20])

The Score	Label index(%)	Symbol	Description
Score I	<5%	—	negative
Score II	6-25 %	+	Low
Score III	26-50 %	++	Moderate
Score IV	51-100 %	+++	High

Plasma cell percentage was estimated in bone marrow aspirate smears, and was reviewed by two specialized hematologist and it was compared to that of the patient's files. All statistical analysis was performed with the SPSS statistical software version 11 and Microsoft Office excel 2003. Comparisons of means were based unpaired Student's t- test (2-tailed). For more than two groups, one- way ANOVA was used and for correlation between two groups, the rules for Pearson correlation was applied. $p<0.05$ was consider statistically significance.

Results:

This study revealed that the mean age of the patients included in this study was 60.08 ± 8.90 years; the range was 38 -74 years and the median was 50 years. The age of 40.5 % of the patients was between 60-69 years , whereas the mean age group of control subject was 56.00 ±7.97 years ,the range was 45-68 years and the median was 52 years. The patients in this study include 19 males (51.35%) and 18 females (48.65 %), male to female ratio 1.05:1, whereas control subjects include 6 males (60%) and 4 females (40 %) male to female ratio 1.5:1 . The results of the immune-staining procedure in MM bone marrow biopsies , was cytoplasmic diffuse brown coloration of the endothelial lined vessels for FVIII-RA (figure 6) and nuclear diffuse brown coloration of the tumor cells for PCNA (figure 5). This study showed that the mean level of MVD using FVIII –RA and PCNA expression in marrow of MM patients were significantly high compared to control cases who had reactive plasmacytosis , as shown in table 2.

Table 2: Comparison of the mean levels of MVD and PCNA expression in multiple myeloma patients with those in control subjects:

Parameters	Patients (n=37) Mean ± SD Range	Control (n=10) Mean ± SD Range	P value
MVD (FVIII-RA)	69.51 ±6.48 10.00- 160.00	6.00 ±1.49 4.00 – 8.00	0.0001
PCNA (% of nuclei)	58.5 ± 4.04 20.00-95.00	3.80 ± 0.13 3.00- 4.00	0.0001

Moreover this study revealed that there was significant positive correlation between both the mean vessel count and PCNA expression (% of nuclei) and the percent of plasma cells infiltrating the bone marrow (r=0.862 , r=0.764 respectively ; P= 0.0001 for both correlations) as shown in figure 1 and 2 respectively.

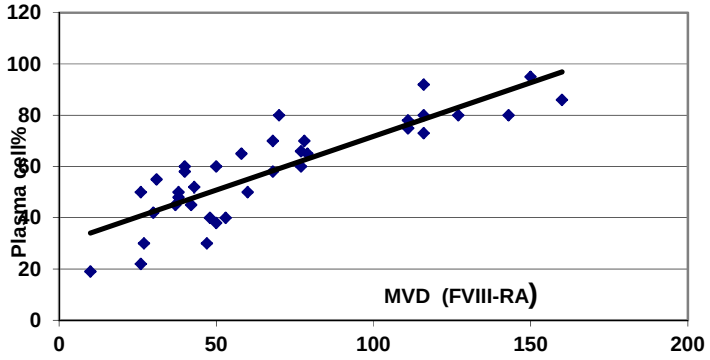

Figure1: The correlation of MVD (FVIII-RA) and Plasma cell percent in bone marrow of patients with multiple myeloma.

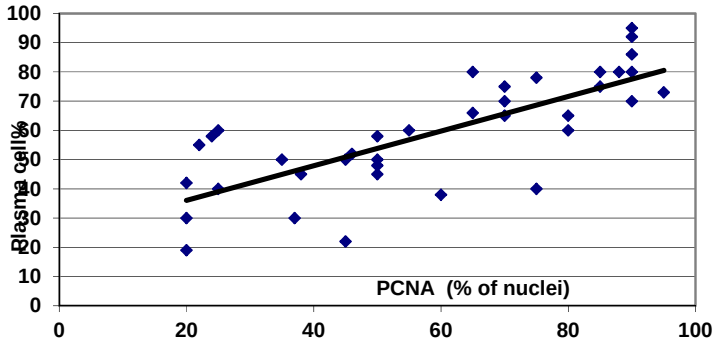

Figure 2: The correlation of PCNA and Plasma cell percent in bone marrow of patients with multiple myeloma.

This study revealed a highly significant positive correlation between PCNA expression and MVD in the bone marrow of patients with MM (r=0.831; P=0.0001) as shown in figure 3. Moreover table 3 , showed that there was significant increase in angiogenetic activity in relation to the PCNA scoring system , so that marked angiogenetic activity was observed in score IV , and PCNA was express significantly only in myeloma patients so that all control cases, who had reactive plasmacytosis in their marrow, had less than 5% PCNA expression or negative score (table 3).

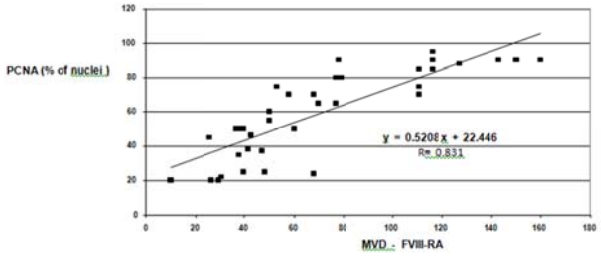

Figure 3: The correlation of PCNA (% of nuclei) and MVD – FVIII-RA in bone marrow of patients with multiple myeloma .

Table 3 : The relation of MVD of PCNA grading according to Alves et al scoring system and MVD in bone marrow of patients with MM .(significant by ANOVA test)

Alves et al scoring system [21]	No	FVIII-related antigen Mean± SD	P value
Score I (0-5%)	-	-	0.0001*
Score II (6-25%)	7	36.29±18.26	
Score III (26-50%)	10	39.70±9.83	
Score IV (51-100%)	20	96.05±34.32	

This study revealed that the percentage of plasma cell infiltrating the bone marrow of multiple myeloma patients increased significantly ($p< 0.001$) in relation to the score of PCNA expression, so that bone marrows with largest number of plasma cell infiltrate (M±SD, 70.6 ± 14.44 %) are grouped in high PCNA score (i.e) score IV, whereas percentages of plasma cell infiltrate in score II and III were 41.42 ± 15.41% and 43.9 ± 11.52% (M±SD) respectively (Figure 4; table 4).

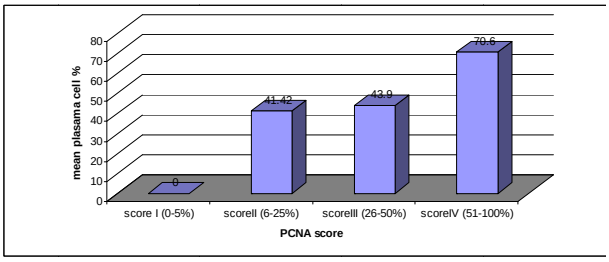

Figure 4: The percentage of plasma cell infiltrating the bone marrow in relation to Alves et al scoring system for PCNA expression (significant by ANOVA test)

Table 4: Distribution of Percentage Plasma cell infiltrate according to Alvas et scoring system.[21]

Score	N	Mean	Std. Deviation	Std. Error	Mini mum	Max imum
II	7	41.4286	15.41490	5.82628	17.00	59.00
III	10	43.9000	11.52003	3.64295	19.00	59.00
IV	20	70.6000	14.77159	3.30303	36.00	95.00
total	37	57.8649	19.61492	3.22467	17.00	95.00

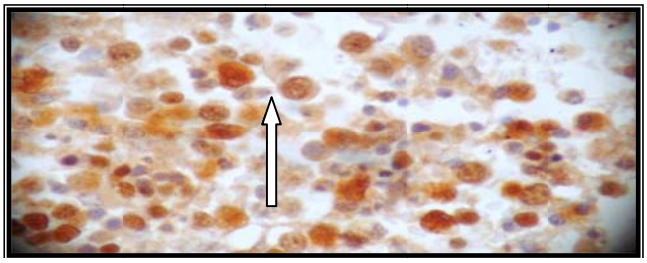

Figure 5: PCNA positive immune stained plasma cells in bone marrow of patient with MM. Arrow directed to stained tumor cell (plasma cell) (100X).

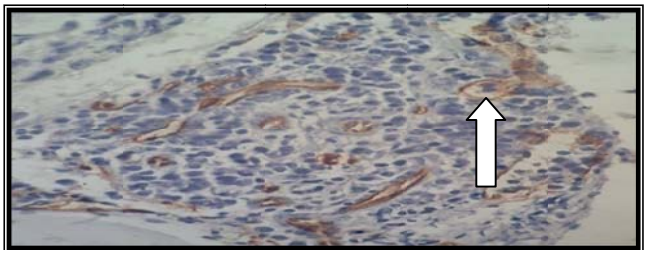

Figure 6: Bone marrow biopsy of MM immune-stained with anti FVIII-related antigen. Arrow directed to stain endothelial lining (100X).

Discussion:

This study revealed that BM angiogenesis as expressed by MVD using immune stained FVIII-related antigen as an endothelial cell marker ,was significantly high in multiple myeloma (MM) patients compared to control subjects (table 1). These results were in agreement with many studies which had revealed that BM angiogenesis progressively increases along the spectrum of plasma cell disorders , from the more benign stage to advanced myeloma , indicating that angiogenesis was related to disease progression .(7,21,22) Increase angiogenesis is considered as a constant hallmark of MM progression and its invasive potential ;It is partly sustained by vascular endothelial growth factor (VEGF) , basic fibroblast growth factor (bFGF) ,hepatocyte growth factor(HGF) and matrix metalloproteinases (MMPs) secreted by the plasma cells.(5,6) Recently Hose D et al assess the expression of 402 angiogenesis-associated genes by Affymetrix DNA microarrays in 466 sample including multiple myeloma cells (MMCs)and normal BM plasma cells (BMPCs). They found that BMPCs express a surplus of proangiogenic over antiangiogenic genes which will induce angiogenesis , whereas MMCs express in addition, aberrant proangiogenic genes and will down regulate some antiangiogenic genes which will further increase the angiogenic stimulus leading to bone marrow angiogenesis in all myeloma patients .(23) The current study revealed that the increased angiogenesis is closely correlated with the percentage of plasma cells in the bone marrow of MM patients (figure 1). It is well known that the percentage of plasma cell correlate with the disease activity and

since Vecca et al studies(7,24) had found that there was five to sixfold increase in angiogenesis as expressed by FVIII-RA MVD in patients with active MM as compared with monoclonal gammopathies of undetermined significance (MGUS) (plasma cell less than 30%) and smoldering myeloma, therefore we may conclude that the progression of plasma cell tumors is constantly accompanied by an increase of bone marrow neovascularization .(7,21,24) This study revealed that PCNA expression is increased significantly in BM of MM patients as compared with control subject as shown in table 2 . This result was in agreement with Pappa *et al* study(14) which stated that PCNA is significantly high in MM and its positively correlates with serum interleukin 10 and 15, thus they had concluded that these proliferative markers can be used to assess disease progression in MM. Similarly PCNA and nucleolar organizer regions (AgNORs), which is a known proliferative marker,were significantly high in MM and they show high expression in patients with immature type MM .(16) PCNA is a cell proliferation marker which plays an important role in nucleic acid metabolism , it is essential for DNA replication and repair, for RNA transcription and for chromatin assembly .Also PCNA interact with cellular proteins involved in cell cycle regulation , progression and check point control , and it therefore maybe considered as an indicator of the proliferative activity of myeloma cells .(8,9) Moreover the highest percentage of bone marrow plasma cell infiltration , were grouped in high PCNA score(i.e) score IV as shown in figure 4 and table 4 .These findings were similar to many studies which revealed that PCNA expression was significantly higher in active pretreated MM compared to post treated cases and control , and the highest expression was seen in the plasmablastic type MM.(16,25,26) Therefore we may postulate that PCNA can be useful in assessing disease progression in MM .This study showed that PCNA expression correlate significantly with bone marrow MVD as shown in figure 3 , table 3 , so that cases with high expression of PCNA had significant high angiogenetic activity .This was in aggrement with Alexandrakis MG et al study which found the pretreatment mean level of PCNA expression , MVD ,plasma cell infilteration and IL-6 were significantly higher than post treatment values and control.(26) From this study we may conclude that the proliferative activity of plasma cell as represented by PCNA expression is closely correlated to angiogenic activity in the bone marrow of multiple myeloma and both markers correlate with percentage of plasma cells infilterating the marrow thus can be used as an auxillary markers for initial evaluation of myeloma bone marrow biopsies .

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