Evaluation of Bone Marrow Microvessel Density in Patients with Aplastic Anemia

Venkatesan Somasundaram¹ · Manvir Singh Tevatia^{2,3} · Abhishek Purohit⁴ · Ankur Ahuja⁵ · Manoranjan Mahapatra⁵ · Seema Tyagi⁵ · Haraprasad Pati⁵ · Renu Saxena⁵

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Abstract Bone marrow microenvironment plays a crucial role in the growth of hemopoietic cells and bone marrow function, which in turn depends on an intact microvasculature. Our study assesses the microvessel density (MVD) in the bone marrow of aplastic anemia (AA) patients, compares with MVD of controls and MVD among the different types of AA. Bone marrow specimens from 60 patients with AA and 17 controls were studied. There were 33 patients with non severe AA (NSAA), 12 patients with severe AA (SAA) and 15 patients with very severe AA (VSAA). MVD was calculated on sections stained immunohistochemically for CD34. The mean bone marrow MVD in AA group was 1.28 ± 0.36 , being significantly lower than that in control group (6.80 ± 1.59 , $p < 0.001$). MVD of SAA and NSAA patients were 1.16 ± 0.35 and 1.49 ± 0.27 , respectively, being significantly different ($p = 0.003$). MVD of VSAA was 0.93 ± 0.25 and the difference with NSAA is significant, however there was no significant difference between SAA and VSAA. Bone marrow MVD is low in AA patients and is likely to have a role in pathophysiology of bone marrow failure.

Proangiogenic agents together with specific therapy might accelerate the recovery of hematopoiesis in AA patients.

Keywords Aplastic anemia · Angiogenesis · CD 34 · Mean vascular density (MVD)

Introduction

Aplastic anemia (AA), the paradigm of the bone marrow (BM) failure syndromes, is a life-threatening disorder characterized by a deficiency of pluripotent hematopoietic progenitor cells with BM aplasia and peripheral pancytopenia [1]. The histological hallmark of the disease is the empty marrow that contains normal amounts of fat cells but is more or less depleted of myelopoietic progenitor cells, erythroid cells, and megakaryocytes [2, 3]. The criteria for diagnosis of AA requires at least two of the following: (1) haemoglobin (Hb) <10 g/dL, (2) platelet count (Plt) $<50 \times 10^3/\mu\text{L}$, (3) absolute neutrophil count (ANC) $<1.5 \times 10^3/\mu\text{L}$ [4]. The severity of disease is graded according to the blood count parameters and bone marrow findings which includes a marrow cellularity of <25 % of normal for that age or absolute level of <50 % with haematopoietic cells representing <30 % of the residual cells and at least two of the following peripheral blood counts: ANC of $<0.5 \times 10^3/\mu\text{L}$, Plt of $<20 \times 10^3/\mu\text{L}$, and/or anemia with a reticulocyte count of $<20 \times 10^3/\mu\text{L}$ [5]. The very severe AA (VSAA) has all criteria as severe AA (SAA), however with neutrophil count of $<0.2 \times 10^3/\mu\text{L}$ and non severe aplastic anemia (NSAA) does not meet the criteria for severe or very severe AA [6]. About 80 % of AA is acquired in nature and the causes of acquired aplastic anaemia are very diverse and is labeled idiopathic when AA occurs without an obvious prior history [7, 8].

✉ Venkatesan Somasundaram
ltcolsvenkatesan@gmail.com

¹ Department of Pathology, Armed Forces Medical College, Pune 411040, India

² Brig Med, HQ Delhi Area, New Delhi, India

³ Department of Pathology, Army College of Medical Sciences, Delhi Cantt., New Delhi, India

⁴ Department of Pathology, All India Institute of Medical Sciences, Jodhpur, India

⁵ Department of Hematology, All India Institute of Medical Sciences, New Delhi, India

As far as the pathogenesis of AA is concerned, there are several indications that AA is due to a stem cell dysfunction. Evidence suggests that a T cell-mediated reaction may be responsible for the stem cell destruction [9]. Furthermore, T cells of patients produce cytokines such as interferon- γ and tumour necrosis factor- α and increased expression of these cytokines has been shown in the BM of patients [10]. These cytokines can suppress stem cell proliferation and induce apoptosis of stem cells [11]. However, the exact mechanisms inducing this immune reaction are unknown [7]. One of the pathogenetic concept proposed in the context of AA, is related to the BM microenvironment, which consists of endosteal cells, macrophages, fat cells, fibroblasts, and microvascular endothelial cells [12]. The microvasculature is an essential component of the microenvironment in diverse organs and has been described as a critical target in various disorders. Likewise, the BM microvasculature is considered to play an important role in hematopoietic neoplasms [13–15]. Few studies have focused on bone marrow angiogenesis (by assessing the microvessel density-MVD) as one of the possible underlying pathogenetic mechanism in AA. It was also suggested that a defect of angiogenesis in BM may play a role resulting in or aggravating hematopoietic aplasia in patients with AA [16–18].

The aims of the present study were to examine BM angiogenesis by assessing MVD in patients with AA and to compare these values with those obtained in control BM and to correlate the MVD values with the severity of AA.

Materials and Methods

This was a prospective study conducted from June 2013 to December 2014 on patients diagnosed to have AA. The study was approved by the Institute's Ethics Committee and performed in accordance with ethics standards laid down in the 1975 Declaration of Helsinki and its later amendments. A written informed consent was obtained from the patients prior to the procedures.

Patients

Total of 60 patients with AA and 17 controls were included in this study. BM aspirate and BM biopsy were performed from the posterior superior iliac spine under local anesthesia and strict asepsis. Apart from a thorough physical examination, other investigations included complete blood count, blood chemistry and viral markers for Hepatitis B, C and HIV after appropriate counseling. The diagnosis of AA was established according to published criteria [4–6]. Based on these criteria, 33 of 60 patients (55 %) were classified as NSAA, 12 patients (20 %) as SAA, and 15 patients (25 %) as VSAA.

Control BM specimens were obtained from two patients with uninvolved marrow in non-Hodgkin lymphoma, three with uninvolved marrow in Hodgkin lymphoma, 12 patients of immune thrombocytopenia. None of these patients had any signs or symptoms of systemic inflammation or lymphoma related B symptoms. 35 patients (58.33 %) received only androgen (Stanozolol 2 mg/kg/day in three divided doses) due to poor socioeconomic status of patients, 22 patients (36.66 %) were treated with androgen and cyclosporin A (CSA) (5 mg/kg/day in two divided doses) while 03 patients (05 %) were given ATG (40 mg/kg for four days) and CSA.

Histology and Immunohistochemistry

In all patients, the BM was examined by routine histology and cytology at diagnosis. Immunohistochemistry (IHC) was performed on paraffin-embedded, formalin-fixed BM biopsy sections using the indirect immunoperoxidase staining technique. Antigen retrieval was done using 0.01 M citrate buffer, pH 6 using microwave. Endogenous peroxidase was blocked by methanol/Hydrogen Peroxide. Slides were incubated with primary antibody for 1 h at room temperature in a dark moist chamber. The CD34 monoclonal antibody (mAb) QBEnd/10 was purchased from Neomarkers Labvision corporation (CA, USA) and used in dilution of 1:70 with 0.05 mol/L Tris-buffered saline (pH 7.0). After washing, slides were incubated with antibody amplifier followed by HRP (Ultravision Quanto detection system, Labvision, CA, USA) in dark for 10 min each. 3,3'-Diaminobenzidine (DAB) (DAB Quanto Kit, Labvision, CA, USA) was used as chromogen. Slides were counterstained in Hematoxylin. The cellularity of the BM was quantified according to published guidelines [19].

Evaluation of MVD

The BM MVD was determined in CD34-stained BM sections essentially as described by Perez-Atayde and et al. [14]. A total of 10 microscopic fields at 20 $\times$ objective (total magnification of 200 $\times$), were examined for the number of blood vessels in the bone marrow [18]. The mean number of blood vessels (MVD per field) is then calculated. The MVD were determined by two independent observers in each case. Large vessels and vessels in the periosteum or bone were excluded because vascularisation is not representative of neoangiogenesis in these areas. Areas of staining with no discrete breaks were counted as a single vessel. Presence of a lumen was not required [20]. The response to therapy was assessed as per the published and accepted recommendations [21]. However the response to therapy is not correlated with MVD in this study.

Statistical Evaluation

The level of significance of MVD between AA and controls was determined by two-sample *t* test. The correlation of MVD with the severity of AA was performed using Chi square test. Results were considered to be significantly different when the *p* value was <0.05.

Results

Demography

The patient age ranged from 4 to 72 years with median age at presentation being 22 years. Majority of the patients were males (40/20); male to female ratio was 2:1. The duration of symptoms ranged from 1 to 60 months (median = 2 months). The commonest symptom being weakness/fatigability noted in all cases followed by bleeding manifestations (66.66 %) and fever (40 %) (Table 1). 53 patients (88.33 %) gave transfusion history at presentation with a range of one to 50 transfusions (median = four). On physical examination, none of the patients had organomegaly or lymphadenopathy. One patient was on follow up for HCV related chronic liver disease. In all other cases, viral markers for Hepatitis B, C and HIV were negative along with normal biochemical parameters. Patients had median Hb of 5.8 g/dL (range 1.2–9.5 g/dL), median ANC of $0.53 \times 10^3/\mu\text{L}$ (range $0.03\text{--}1.4 \times 10^3/\mu\text{L}$) and median Plt of $13 \times 10^3/\mu\text{L}$ (range $10\text{--}63 \times 10^3/\mu\text{L}$).

Evaluation of Angiogenesis in Patients with AA Using CD 34 Immunohistochemistry

Patients with AA exhibited reduced BM cellularity which ranged from 5 to 20 %. There were 33 patients with NSAA, 12 patients with SAA and 15 patients with VSAA. As assessed by CD34 IHC, the mean BM MVD was significantly lower in patients with AA compared to controls (Controls 6.80 ± 1.59 vs. AA 1.28 ± 0.36 , $p < 0.001$) (Figs. 1, 2). A decreasing trend in mean MVD was noted from NSAA to SAA to VSAA (Table 2). The difference in mean MVD between NSAA and SAA is statistically significant ($p = 0.003$). Similarly a statistically significant difference was noted in mean MVD between NSAA and VSAA ($p < 0.001$), however the mean MVD difference between SAA and VSAA is not significant ($p = 0.119$).

Treatment and Follow Up

35 patients (58.3 %) received only androgen, 22 patients (36.66 %) were treated with androgen and CSA, while 03 patients (05 %) were given ATG and CSA. The patients

Table 1 Base line characteristics of study population

<i>Age (years)</i>	
Median	22
Range	4–72
<i>Gender</i>	
Male	40 (66.67 %)
Female	20 (33.33 %)
<i>Hb (g/dL)</i>	
Median	5.8
Range	1.2–9.5
<i>ANC ($10^3/\mu\text{L}$)</i>	
Median	0.53
Range	0.03–1.4
>0.5	33 (55 %)
<0.5	12 (20 %)
<0.2	15 (25 %)
<i>Plt ($10^3/\mu\text{L}$)</i>	
Median	13
Range	10–63
<20	38 (63.33 %)
≥20	22 (36.67 %)
<i>Bone marrow cellularity (%)</i>	
Median	05
Range	05–20
<i>HBsAg</i>	
Positive	0 (0 %)
Negative	60 (100 %)
<i>Anti-HCV</i>	
Positive	1 (1.67 %)
Negative	59 (98.33 %)
<i>Anti-HIV</i>	
Positive	0 (0 %)
Negative	60 (100 %)

had a median follow up of 6 months (range 1–17 months). Response was assessed in only those patients who had a minimum follow up of 6 months. Of those cases, four had partial response (PR) while four patients had complete response (CR) at the end of 6 months follow up. Of the patients who had response (PR and CR) to therapy, one patient was given only androgen (as the patient could not afford any other form of treatment and not as a treatment of choice), six patients received androgen and CSA while one patient was treated with CSA and ATG.

Discussion

The acquired AA is caused by different pathophysiological conditions, which include decreased hematopoietic stem cell (HSC) number, impaired HSC function, increased bone marrow cellular apoptosis level, functional and structural

Fig. 1 Microvessel density (MVD) of bone marrow biopsy immunostained with CD 34, showing lower MVD in patients as compared to controls. **a** MVD in control ($\times 200$). **b** MVD in control ($\times 400$). **c** MVD in case ($\times 200$). **d** MVD in case ($\times 400$)

Fig. 2 Box and whisker plot showing MVD in controls and cases. The horizontal lines represent 90th, 75th, 50th, 25th and 10th percentiles. Circles are outliers

defects in the bone marrow hematopoietic microenvironment and several microenvironmental components [22]. Studies have highlighted the role of microenvironment in pathogenesis of aplastic anemia [23]. And similarly few studies have correlated the role of angiogenesis which is a part of microenvironment, in pathogenesis of aplastic

Table 2 Mean MVD count in different subtypes of AA

AA type	Number of cases	Mean MVD count
NSAA	33	1.49 ± 0.27
SAA	12	1.16 ± 0.35
VSAA	15	0.93 ± 0.25

anemia by effectively quantifying the microvessels and comparing the same with that of controls [16, 18, 20, 24, 25].

AA is a heterogeneous disease not only in terms of its pathogenesis but also in severity and response to therapy [26]. In this study, patients with NSAA, SAA, and VSAA along with controls were examined. The mean MVD of AA cases differed significantly from controls. Also the mean MVD showed a decreasing trend from non-severe to very severe AA. There was significant difference in the mean MVD between NSAA and SAA, NSAA and VSAA. However we could not demonstrate significant difference between the mean MVD count between SAA and VSAA even though the mean MVD count of VSAA was lower than SAA. These findings could suggest that there is decreasing angiogenesis in the bone marrow as the severity

of AA increases. The decrease in angiogenesis is possibly due to reduction in cytokine levels in particular Vascular endothelial growth factor (VEGF), in SAA and VSAA. The results of our study was in agreement the studies published earlier [16, 18, 20].

Ji et al. reported reduced bone marrow angiogenesis in AA patients compared to controls. According to the authors, defect of angiogenesis in bone marrow may play a role resulting in or aggravating hematopoietic aplasia in patients with AA [16]. Fureder et al. demonstrated the reduced expression of VEGF in marrow components in addition to low mean MVD in cases of aplastic anemia. However their study did not reveal any significant difference in MVD count among the subcategories of AA. They also demonstrated the significant increase in MVD in those AA cases who showed response, reiterating the role of angiogenesis in the pathobiology of AA [18]. Gupta et al. [20] in their study comprising 25 cases of AA also demonstrated similar results and suggested that probably an abnormal (decreased) growth and function of microenvironmental cells as a result of decreased angiogenesis in the BM may contribute to the development of BM aplasia in patients with AA. Our study is unique in the sense it is one of the largest, to study the MVD in 60 cases of AA, previous largest was by Zhang et al. [17] with 51 cases of AA.

To conclude, our study shows that AA is associated with reduced BM angiogenesis. Upon quantification of angiogenesis, it is seen that the BM MVD reduces with the severity of the disease. This observation advances our knowledge about the role of microenvironment in AA and may have implications for new concepts relating to the therapy and outcome of this disease.

Future Perspectives

The treatment options available at present for AA include hematopoietic stem cell transplantation and ATG with CSA. The knowledge about microenvironment and angiogenesis may guide future research towards looking at the option of using pro-angiogenic drugs in treatment of AA. The promotion of MVD in bone marrow and the improvement of microenvironment may induce hemopoiesis and create a new way to cure aplastic anemia.

Compliance with Ethical Standards

Conflict of interest None.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical

standards. The study was also approved by the institutional ethical committee.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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