



ARAŞTIRMA / RESEARCH

Effects of bone marrow fibrosis and angiogenetic structure on autologous hematopoietic stem cell engraftment

Kemik iliği fibrozis ve anjiogenezinin hematopoetik kök hücre engraftmanı üzerine etkisi

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Abstract

Purpose: Hematopoietic stem cell (HSC) engraftment is influenced by many factors. We investigated the effects of bone marrow fibrosis and angiogenetic structure on engraftment in patients with hematological malignancies.

Materials and Methods: Data were collected from 34 patients (20 males and 14 females) who underwent autologous HSC transplantation. Bone marrow myelofibrosis was graded from 0 to 3, angiogenesis was quantified using a stereological method in the most recent bone marrow biopsy before the transplantation. Patients were categorized into two groups according to intensity of angiogenesis parameters.

Results: Half of the patients had fibrosis and majority had multiple myeloma (73.5%). Eleven patients had grade 1, six had grade 2 myelofibrosis. The engraftment day (ED) for platelets and erythrocytes was significantly different between the grade 2 fibrosis and non-fibrosis groups. VSD and NVES levels were significantly higher in the grades 1 and 2 fibrosis groups than the no fibrosis group. While the overall survival time was shorter in the grade 2 fibrosis group than the others, the difference was not statistically significant.

Conclusion: Bone marrow fibrosis was found to be independent risk factor. It may have a negative effect on platelet and erythrocyte engraftment time of autologous transplantation process but this effect does not influence survival.

Key words: Bone marrow fibrosis, angiogenesis, autologous transplantation, multiple myeloma.

Öz

Amaç: Birçok faktör hematopoetik kök hücre (HKH) engraftmanını etkiler. Bu çalışmada hematolojik kanseri olan hastalarda kemik iliğindeki myelofibrozis ve anjiogenezinin engraftman üzerine etkisi araştırılmıştır.

Gereç ve Yöntem: Ototolog kök hücre nakli yapılan 34 hasta (20 erkek, 14 kadın) verileri değerlendirildi. Nakilden önceki son kemik iliğindeki fibrozis 0-3 arasında derecelendirildi, anjiogenezis stereolojik metod ile ölçüldü. Hastalar anjiogenezis parametrelerinin yoğunluğuna göre de iki gruba ayrıldı.

Bulgular: Hastaların çoğunluğu (%73.5) multipl myelom idi ve yarısında fibrozis saptandı. On bir hasta derece 1, 6 hasta derece 2 fibrozise sahipti. Trombosit ve eritrosit engraftman günleri açısından derece 2 fibrozisi olan grupta fibrozis saptanmayan grup arasında istatistiksel anlamlı farklılık vardı. Derece 1 ve 2 fibrozis gruplarında fibrozisi olmayanlara göre VSD ve NVES düzeyleri anlamlı olarak yüksekti. Toplam yaşam derece 2 fibrozisi olan grupta daha düşük olmakla birlikte istatistiksel anlamlılık yoktu.

Sonuç: Kemik iliği myelofibrozisinin bağımsız bir risk faktörü olduğu saptanmıştır. Bunun toplam yaşamı etkilemeden otolog nakil sürecinde trombosit ve eritrosit engraftmanı üzerine negatif etkisi olabilir. Fibrozisin derecesi ile doğru orantılı olarak artmış anjiogenezis arasındaki ilişkiyi açıklamak için ileri çalışmalar gereklidir.

Anahtar kelimeler: Kemik iliği fibrozisi, anjiogenezis, otolog transplantasyon, multipl myelom.

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INTRODUCTION

One of the most important criteria for successful autologous stem cell transplantation is overcoming aplasia of bone marrow that occurs due to the conditioning regimen. Recovery of bone marrow and reconstitution of hematopoiesis with re-infused autologous stem cells is called engraftment.

The period of engraftment is expected to be 7–21 days after transplantation. If the duration is > 21 days, it indicates an engraftment delay. Engraftment failure is defined as > 42 days¹⁻³. Bone marrow consists of hematopoietic and stromal compartments composed of reticular cells, adipocytes, and osteogenic bone cells near the surfaces of vascular endothelial cells, vascular endothelial smooth muscle cells, and macrophages^{4,5}. The “hematopoietic stem cell niche” was first defined by Schofield in 1978. Stem cells are located in this micro-anatomic region of bone marrow where they are blocked from differentiating or self-renewing. The stem cells are supported by adjacent stromal cells.

Hematopoietic stem cells (HSCs) and hematopoietic progenitor cells are distributed randomly in the bone marrow but are rather close to the bone endosteum⁶⁻⁸. Recent studies have demonstrated that they accumulate around blood vessels^{9,10}. The differentiation and maturation processes of bone marrow HSCs were described by Shacney in 1975¹¹. The area where HSCs are located is rich in vascularization and closer to the centre of bone marrow. Shacney also noted that undifferentiated cells are found throughout the endosteum.

The endosteal bone marrow sinusoidal network zone defines a different anatomical and functional structure and was adapted to the “vascular niche” concept. Ultrastructural studies have shown that differentiated HSCs are located closer to bone marrow microvascular structures compared to immature HSCs^{12,13}. HSC engraftment is a complex process that entails the collection of circulating bone marrow HSCs throughout the microvascular structure and subsequent trans-endothelial migration to the bone marrow hematopoietic cords. These events are referred to as HSC homing. Then, stem cells settle to a specific region known as the niche, which defines lodgment¹⁴.

These findings indicate that the interaction between progenitor cells and stromal cells is a critical

determinant of the course of HSC maturation. Many factors that affect engraftment kinetics have been examined in patients undergoing autologous peripheral blood stem cell transplantation; however, the debate over engraftment process continues.

In the present study, we investigated the effects of bone marrow fibrosis and angiogenesis on engraftment.

MATERIAL AND METHODS

Patients

This study was planned as a single-center, cross-sectional and retrospective study. Patients who underwent autologous peripheral stem cell transplantation (PSCT) over 6 years were enrolled in this retrospective study. Study material was collected from the records starting on January 2003 and ending at December 2008. Pre-transplant bone marrow biopsies obtained from 34 consecutive patients (20 males and 14 females) were examined. Sections from paraffin blocks were placed on poly L-lysine slides, preserved in the Pathology Department archive, and were evaluated by two hematopathologists. Marrow structure was examined fibrotic and angiogenetic structure. Patients were grouped according to the degree of fibrosis.

Diagnosis, disease stage, treatment, response, total number of CD34⁺ peripheral stem cells (PSCs) collected, mobilization regimen, pre-transplant disease status, and the last date of contact or date of death were recorded from the patient files. Neutrophil, platelet and erythrocyte engraftment day were noted.

Primary endpoint was defined as neutrophil, platelet and erythrocyte engraftment time associated with bone marrow structure. Secondary endpoint was the effect of marrow structure on survival.

Ethics committee approval was obtained from the Institutional Ethics Committee prior to the study. Informed consent was obtained from all individual participants included in this study.

Definitions

Neutrophil engraftment day (ED) was defined as the first day that the neutrophil count remained $\geq 500 \times 10^6/L$. Platelet engraftment had occurred when the platelet count was $> 20 \times 10^9/L$ for 7

days without a transfusion. Erythrocyte engraftment required an interval of 3 weeks without a blood transfusion¹.

Angiogenesis was evaluated by using the CD34 (NeoMarkers, Fremont, CA, USA) antigen streptavidin-avidin-immunoperoxidase method. Stereological measurements were performed to determine the vascular density visible with CD34. Number of vessels per unit stroma (NVES) and vascular surface density (VSD) per tumor tissue volume were calculated according to the following formulae¹⁵.

$$VSD = \frac{\sum ln.2.121}{Istr.Lr} \quad NVES = \frac{N.121}{Istr}$$

The sections were fixed at 100× magnification for light microscopy and reflected on a camera (CCD; Sony, Tokyo, Japan) and monitor (Sony Trinitron). A 525× magnified image was also obtained. The image was placed on a transparent monitor composed of 121 points formed by 11 horizontal and 11 vertical test lines. The tumor tissue was contained within the borders of the transparent area, and the number of junction points where the vessels crossed test lines (In) were noted as the total vessel count (N) whether they crossed or not. Vv (str) indicates the stromal volume ratio above the test line and was determined by the point-counting method. Vv (str) is the point count per stroma

divided by 121. The same process was repeated in 10 randomly selected fields. Lr (constant number), 10.34; Istr, number of crossed horizontal and vertical lines in the specified stromal field.

Myelofibrosis was graded based on the European Consensus Report of Bone Marrow Fibrosis grading of 0–3 in sections treated with reticulin silver and Masson’s trichrome stains¹⁶. All patients received 2.4 g/m² cyclophosphamide and a granulocyte colony stimulating factor protocol to mobilize the stem cells. The conditioning regimen was melphalan for multiple myeloma and BEAM (carmustine, etoposide, ARA-C, and melphalan) for lymphoma.

The patients were classified into three groups according to CD 34 level, fibrosis based on literature. Furthermore, patients were classified into two groups with regard to intensity of angiogenetic parameters.

Statistical analysis

Continuous variables between the groups were analysed using the Mann–Whitney U or Kruskal–Wallis tests. Correlations between continuous variables were detected using Spearman’s correlation test. Linear regression models were performed to determine the independent risk factors related with neutrophil, platelet, and erythrocyte engraftment days.

Table 1. Pre-transplantation characteristics of the patients

Characteristic	Patients #	%
Diagnosis		
Multiple Myeloma	24	70.6
Non-Hodgkin lymphoma	5	14.7
Hodgkin lymphoma	4	11.8
Plasmacytoma	1	2.9
Remission status		
VGPR	7	20.6
PR	23	67.6
SD	2	5.9
ResD	1	2.9
RefD	1	2.9
No. CT regimens		
0–1	20	58.8
> 1	14	41.2
Radiotherapy		
Yes	18	52.9
No	16	47.1

No. CT: number of prior chemotherapy regimens, VGPR: Very good partial remission, PR: partial remission, SD: stabile disease, ResD: residual disease, RefD: refractory disease

The predictors of time to transplantation and overall survival rate were analysed using the Kaplan–Meier method and compared to the Mantel log-rank test. Results are reported as median (range), number (n), and percent (%). A p-value < 0.05 was considered significant. Statistical analyses were performed using the SPSS v 20.0 software package (SPSS Inc., Chicago, IL, USA).

RESULTS

The median age of the patients was 50 years (range, 17–70 years). Most patients were diagnosed with multiple myeloma (73.5%), and disease status was partial remission in 67.6%. Of all patients, 55% had received only one line of chemotherapy (CT) before transplantation and 45% had received two or more lines of CT. The pre-transplantation characteristics of the patients are shown in Table 1. The patients were divided into three groups based on CD34⁺ cell level. The CD34⁺ cell levels were < 5 × 10⁶/kg, 5–9.9 × 10⁶/kg, and ≥ 10 × 10⁶/kg in groups 1, 2, and 3 respectively. No differences were found in terms of the numbers of neutrophil, platelet, or erythrocyte EDs among these three groups (Table 2). There were no statistically significant differences among CD 34⁺ groups with regard to age, disease status, receiving radiotherapy, fibrosis and angiogenetic parameters (p>0,05 for all)

A correlation analysis was performed between for the numbers of neutrophil, platelet, and erythrocyte EDs in each CD34⁺ groups. As a result, no significant correlation was detected between CD34⁺ cell level and the numbers of neutrophil or erythrocyte EDs. A trend to negative correlation was observed between CD34⁺ cell level and the number of platelet EDs (r=−0.32, p = 0.016).

The numbers of patients in fibrosis groups 0, 1 and 2 were 17 (50%), 11 (32.4%), and 6 (17.6%), respectively. No patient had grade 3 fibrosis. A significant difference was detected in the number of platelet and erythrocyte EDs between the groups (p=0.037 and p=0.010, respectively). The median numbers of platelet and erythrocyte EDs were similar in patients with grades 0 and 1, but these durations were significantly longer in the grade 2 fibrosis group than in the other groups. However, the number of neutrophil EDs was not different (p=0.257) among the three fibrosis groups. The median levels of the angiogenesis parameters (VSD and NVES) increased as the degree of fibrosis

increased. Significant differences were detected in the VSD and NVES among the fibrosis groups (p=0.034 and p=0.028, respectively). The median VSD and NVES levels were similar in patients with grades 1 and 2 fibrosis, but the median levels were significantly lower in grade 0. A post-hoc test was not applied between the fibrosis subgroups due to insufficient sample size.

No correlations were found between VSD, NVES, and the numbers of neutrophil, platelet, and erythrocyte EDs (r<0.20 for all). Angiogenesis was evaluated as number of micro vessel count per field (NVES) and vascular surface density (VSD) per tumor tissue volume. Median was detected as 35,4 (min:4,02 max:112,3) mm² for VSD and 92,8 (min:10,3- max:325,7) mm¹ for NVES. Patients were categorized into two groups according to median levels of VSD and NVES. VSD levels of ≤35,4 mm² and >35,5 mm² were classified as Group 1 and Group 2, respectively. Similarly, NVES values of ≤92,9 mm² and ≥93 mm² were classified as Group 1 and Group 2, respectively. A statistically significant difference was not detected neither among VSD groups nor NVES groups in terms of neutrophil, platelet and erythrocyte engraftment days (Table 2).

Mean overall survival was 53.7 months (95% confidence interval [CI], 40.5–66.9 months) and mean time to transplantation was 34.0 months (95% CI, 24.7–43.3 months) (Table 3). Overall survival was lower in grade 2 compared to grade 1 patients (28.3 vs. 58.4 months). Similar results were observed among patients with fibrosis grades 1 and 2 for time to transplantation (34 vs. 16 months, respectively) (Table 3, Figure 1). Cox-regression models were not performed to determine the independent risk factors related with neutrophil, thrombocyte and erythrocyte engraftment days due to insufficient sample size.

Three linear regression models were performed: neutrophil, platelet, and erythrocyte engraftment days were used as dependent variable; VSD, CD 34⁺ cell amount measured as continuous variables; fibrosis and disease status at pre-transplantation were used as independent variables in the separately models. The results of linear regression models showed that fibrosis was found to be significantly independent factor related with platelet and erythrocyte engraftment days (p=0.037 and p <0.001 respectively).

Table 2. The neutrophil, thrombocyte and erythrocyte engraftment days in several groups

Median (min-max)	Neutrophil engraftment days	Thrombocyte engraftment days	Erythrocyte engraftment days
CD34 ⁺ cell groups			
1 (n:12)	11.5 (9-19)	16.5 (10-35)	13.0 (9-35)
2 (n:11)	11.0 (9-12)	12.0 (10-24)	14.0 (9-32)
3 (n:11)	10.0 (8-13)	12.0 (8-18)	12.0 (3-27)
p value	0.148	0.247	0.714
Fibrosis grade groups			
0 (n:17)	10 (8-14)	12 (9-34)	11 (3-30)
1 (n:11)	11 (9-19)	12 (8-35)	12 (9-35)
2 (n:6)	11.5 (10-14)	18 (14-24)	22.5 (14-32)
p value	0.257	0.037	0.010
Fibrosis groups			
No fibrosis (n:17)	10 (8-14)	12 (9-34)	11 (3-30)
Fibrosis (n:17)	11 (9-19)	15 (8-35)	14 (9-35)
p value	0.122	0.071	0.074
VSD groups			
1 (n:17)	11 (9-14)	12 (8-35)	12 (3-35)
2 (n:17)	11 (8-19)	15 (10-23)	14 (7-27)
p value	0.623	0.376	0.615
NVES groups			
1 (n:17)	11 (9-19)	12 (8-35)	12 (3-35)
2 (n:17)	11 (8-14)	14 (10-23)	14 (7-27)
p value	0.806	0.972	0.603

NVES: Micro vessel count per field, VSD: vascular surface density per tumor tissue volume.

While vascular surface density per tumor unit was found to be significantly independent factor for erythrocyte engraftment day ($p=0.025$), CD 34⁺ cell amount was found to be significantly independent factor for platelet and neutrophil engraftment day ($p=0.004$ and $p=0.020$ respectively).

DISCUSSION

It is quite important to determine the hematopoietic engraftment rate and long-term continuity of engraftment following peripheral stem cell transplantation. Mortality and morbidity risks, transfusion and antibiotic needs, hospital stay duration, and economic costs of engraftment make this condition critical.

The number of CD34⁺ cells in a graft is one of the factors affecting its kinetics. The required number of HSCs for autologous transplantation is $2.5-5 \times 10^6$ CD34⁺/kg according to some authors¹⁷⁻²⁰. However, Maunier et al.²¹ found that the optimal level was a minimum of 5×10^6 CD34⁺/kg in a study that investigated the influence of CD34⁺ cell number (before cryopreservation and when melted)

on long-term hematologic reconstruction following autologous stem cell transplantation in patients with lymphoma. Furthermore, Hohaus et al.²² showed that platelet engraftment is faster in patients with lymphoma, if the number of infused CD34⁺ cells is $> 5 \times 10^6$ /kg. Our results show a similar number of EDs for neutrophils, platelets and erythrocytes regardless of the CD34⁺ cell count.

There are some evidences that marrow fibrosis can delay in stem cell engraftment time in patients with acute and chronic leukaemia. Soll et al.²³ investigated the role of myelofibrosis in 203 patients with myelofibrosis and 203 controls. No difference in the number of neutrophil EDs was reported, but platelet engraftment was delayed 3 days in the myelofibrosis group. This result was a 7-day interval in the severe myelofibrosis group (grades 3-4) for platelet engraftment. Those authors also found a delay of 2 days for erythrocyte engraftment; however, the transfusion requirement was not different. In contrast, Scott et al.²⁴ investigated patients with acute myeloid leukaemia and advanced myelodysplastic syndrome with multi-lineage dysplasia (471 patients: 113 with myelofibrosis and

358 without fibrosis), and detected severe fibrosis in 28% of the patients. Neutrophil engraftment was observed in 98 of 113 patients, and the median ED

was day 28 (range, 10–80 days) in these patients and day 17 (range, 10–33 days) in the cases without fibrosis.

Table 3. Survival analyses in fibrosis and angiogenesis groups

	Mean Overall Survival (month)	Mean Time to Transplantation (month)	Total n / Events n (% of alive)
Fibrosis grade groups			
0 (n:17)	65.6	41.1	17/5 (70.6)
1 (n:11)	58.4	34.9	11/4 (63.6)
2 (n:6)	28.3	16.4	6/3 (50.0)
p value*	0.469	0.364	
Fibrosis groups			
No fibrosis (n:17)	65.6	41.1	17/5 (70.6)
Fibrosis (n:17)	50.2	29.3	17/7 (58.8)
p value*	0.801	0.452	
VSD groups			
1 (n:17)	61.565	36.8	5/12 (70.6)
2 (n:17)	45.036	18.3	7/10 (58.8)
p value*	0.409	0.258	
Overall	53.7	34.0	34/12 (64.7)

*: Log rank

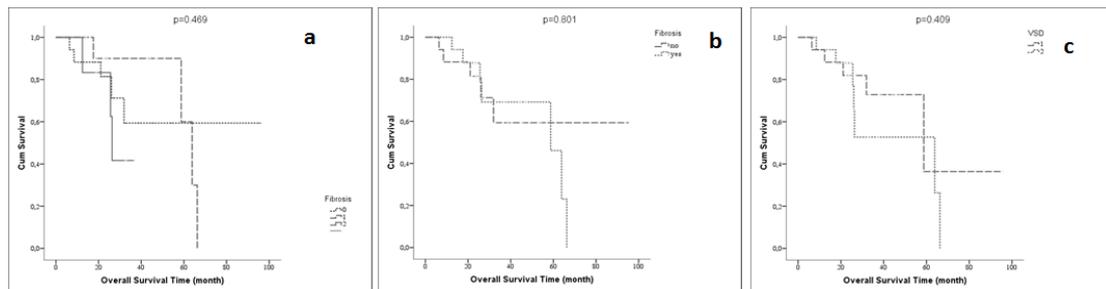


Figure 1: Kaplan Meier curves according to fibrosis grade groups (a), fibrosis groups (b) and VSD groups (c).

These results indicate a significant delay in the fibrosis group ($p \leq 0.0001$). We found a delay in platelet and erythrocyte engraftment time in patients with fibrosis. Our results reveal that the engraftment delay becomes more prominent as fibrosis grade increases. Although angiogenesis processes of many diseases have been investigated, particularly those for solid tumours, their influence on transplantation kinetics have not been sufficiently evaluated. Some evidence of vascular support for the malignant process can be seen in the bone marrow of patients with hematologic cancers, as in other solid organ tumours^{25,26}. Angiogenesis was evaluated in the bone marrow of patients with polycythemia vera, chronic myeloid leukaemia and myelofibrosis, and the results showed that neovascularisation increased in the patients with myeloproliferative diseases. The vascular density of bone marrow was statistically

significant in patients with myelofibrosis (5.9 ± 2.1 and 14.4 ± 5.5 , respectively, $p < 0.001$).

According to a novel pathogenic hypothesis for myelofibrosis with myeloid metaplasia, megakaryocytes and/or monocytes clonally increase due to abnormal levels of cytokines released from impaired bone marrow stroma²⁷ leading to collagen fibrosis and new bone formation. This impaired stromal microenvironment also serves as a source of angiogenic cytokines, such as increased expression of vascular growth factor by megakaryocytes²⁸. Our results show that only erythrocyte engraftment is influenced by the VSD. This result is consistent with the hypothesis mentioned above. We suggest that angiogenesis may help alleviate the negative effects of fibrosis, although no direct effect on engraftment was shown.

Regarding survival, one study investigated the influence of fibrosis on ED and overall survival in 19 patients with fibrosis who underwent autologous transplantation. There was no difference in overall survival between the groups with and without fibrosis and the results according to fibrosis grade were not evaluated in that study²⁹. We showed no statistically significant difference in survival by grade of fibrosis.

In conclusion, bone marrow fibrosis accompanying multiple myeloma and lymphoma was detected to be an independent risk factor which negatively affects platelet and erythrocyte engraftment time, but this effect does not influence overall survival. Preliminary findings in this study also revealed angiogenic changes in the bone marrow occurred dependent from intensity of marrow fibrosis. The observation that VSD (part of angiogenic structure) is an independent risk factor for erythrocyte engraftment may be an important preliminary data for the studies that would be conducted with patients group with larger sample size. For explaining the exact mechanism, future studies are needed.

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