Original research paper

Angiopoietins in the bone marrow microenvironment of acute lymphoblastic leukemia

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Objective: Angiogenesis have implications in leukemia biology. Angiopoietin 1 (Ang 1) is an angiogenic cytokine which is essential in survival and proliferation of endothelial cells. Angiopoietin 2 (Ang 2) promotes dissociation of pericytes and increases vascular permeability and stromal derived factor 1 alpha (SDF 1α) which is a key player in stem cell traffic in the bone marrow (BM), has stimulating effects on angiogenesis as well. Here, we investigated the role of the leukemic BM microenvironment and specifically, the role of SDF 1α-CXCR4 and Ang 1/Ang 2–Tie 2 axes.

Methods: Here, Ang 1, Ang 2, and SDF 1α levels were measured in the BM plasma and in supernatants of mesenchymal stem/stromal cells (MSCs) of patients with ALL and compared with those of healthy controls.

Results: The results showed that at diagnosis, BM plasma levels of Ang 1 and SDF 1α were significantly low and Ang 2 was high when compared to control values. Remission induction was associated with an increase in Ang 1/Ang 2 ratio and SDF levels in BM plasma.

Discussion: The results suggest that BM microenvironment and leukemic cell–stroma interaction influences the secretion of Ang 1, 2 and SDF 1α, thus, may affect both angiogenesis, homing and mobilization of leukemic blasts.

Keywords: Acute lymphoblastic leukemia, Children, Angiopoietins, SDF 1α, Bone marrow microenvironment, Ang 1/Ang 2 ratio

Introduction

Acute lymphoblastic leukemia (ALL) is a malignant disorder of lymphoid progenitor cells with a peak prevalence in preschool ages.¹ Recent studies revealed that modifications within the bone marrow (BM) microenvironment play a major role in development and progression of leukemia.² The BM is a unique dynamic network of growth factors, cytokines, progenitor cells, stem cells, and stromal cells, providing a permissive environment for leukemogenesis and progression.² Crosstalk between leukemic cells and BM stroma including mesenchymal stem/stromal cells (MSCs) is critical in determining the outcome of leukemia. As well as maintaining physiological hematopoiesis, MSCs also have a role in providing suitable microenvironment for the proliferation of tumoral cells. In the last decade, there is an emerging concept that MSCs are one of the key players in chemotherapy resistance.³

Angiopoietin (Ang)–Tie system is a predominant regulator of vascular integrity. Angs are ligands of the Tie 2 receptor. Ang 1 acts as an agonist and activates the Tie 2 signaling pathways, and Ang 2 acts as an antagonist specifically blocking the Ang 1 dependent activation of the same pathways.⁴ Current data about contribution of Ang–Tie system to tumor angiogenesis are generally obtained from solid tumors.⁵ Ang 1 which is expressed on perivascular cells like pericytes, vascular smooth muscle cells, fibroblasts, osteoblasts, and tumoral cells⁶–⁸ is essential for the survival and proliferation of endothelial cells as well as for the formation and stabilization of new vessels.⁵ Ang 1–Tie 2 interaction keeps vascular endothelial cells and
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Hematopoietic stem cells (HSCs) in the quiescence phase. It is supposed that in a similar way Ang 1 is important in keeping leukemic stem cells in osteoblastic niche where they remain in quiescence phase. Ang 2 which is expressed on endothelial cells takes place when endothelium is activated and it functions as an Ang 1 antagonist promoting the dissociation of pericytes from pre-existing vessels and increase in vascular permeability. Expression of Angs is upregulated in several tumors. Recent studies have shown that the ratio of Ang 1/Ang 2 has a critical role in the outcome of solid tumors and a decrease in this ratio in favor of Ang 2 is a marker of poor prognosis in many cancer types.

Stromal Derived Growth Factor 1α (SDF 1α/CXCL12) is mainly expressed by MSCs in various organs and tissues, such as the liver, lungs, lymphatic tissues, and BM. SDF 1α-CXCR4 (receptor for SDF 1) signaling pathway is well known for its role in homing of HSCs into BM and regulation of primary tumor growth and metastasis. SDF 1α also regulates the process of homing and engraftment of leukemic stem cells into the BM as well as it acts in leukemic cell survival, proliferation, differentiation. Inhibition of SDF 1α-CXCR4 leads to the differentiation and mobilization of leukemic blasts making them more sensitive to chemotherapy. SDF 1α which plays a major role in governing stem cell traffic and all biological functions related to stem cell in BM microenvironment, also has important roles in angiogenesis. SDF 1α promotes angiogenesis in different ways such as functioning in homing and proliferation of endothelial progenitor cells in the tumoral region, inducing the secretion of matrix metalloproteinases which degrades extracellular matrix and activates the growth factors like basic fibroblast growth factor (bFGF) and Interleukin 8 which is also an angiogenic factor.

Both BM stroma and leukemic blasts promote angiogenesis in ALL. Up to date, contribution of angiogenic factors such as vascular endothelial growth factor and bFGF to leukemogenesis have been investigated in several trials. However the effect of Angs on pediatric ALL has not been studied and there are very limited data concerning stromal derived factor 1 alpha (SDF 1α) in ALL yet. Here, we investigated the role of the leukemic BM microenvironment and specifically, the role of SDF 1α-CXCR4 and Ang 1/Ang 2–Tie 2 axes. In this study, the levels of multiple angiogenic factors such as Ang 1, Ang 2, and SDF 1α were measured in BM plasma and supernatants of MSCs representing BM microenvironment and compared with healthy controls. Considering the favorable role of early response to treatment in ALL the changes in the levels of angiogenic cytokines and Ang 1/Ang 2 ratio before and after the remission induction therapy were assessed. In order to elucidate the contribution of MSCs to the release of the cytokines, MSCs supernatants were also tested for those parameters.

Patients and methods

Samples

In this prospective controlled trial, 20 newly diagnosed pediatric patients with ALL were included. Fifty one patients attended to Ankara Children Hematology Oncology Hospital between April 2011 and February 2013 and 20 of them whose marrow sample was available for the study were selected. Patients with ALL who had additional diseases affecting BM (Fanconi Aplastic anemia), relapsed ALL or previous stem cell transplantation were excluded. Eight age and sex matched healthy BM donors were selected as control group. After receiving Institutional Ethical Board approval, written informed consent was obtained from all participants’ parents/guardians prior to their inclusion in the study.

The patients were diagnosed, classified, and treated according to the ALL-BFM 2000 protocol. BM samples were collected at the diagnosis and day +33 of the remission induction therapy. Blast percentages were determined microscopically using May–Grünwald Giemsa staining.

Plasma of the BM samples were isolated by centrifugation at 2500 rpm for 15 minutes and then stored at −80°C.

MSCs harvest and culture

Leukemic cells or mononuclear cells from controls were isolated by Biocoll separating solution (density: 1.077 g/ml) (Biochrom, Berlin, Germany) density gradient centrifugation as previously described. In order to expand MSCs, BM mononuclear cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 1-glutamin and 10% fetal bovine serum (FBS). The cultures were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO2 and medium was changed every 3 days. Adherent cells were harvested by Tripsin–EDTA enzyme at 70–80% confluence (passage 0) (Fig. 1A). This procedure was repeated until passage 2 (P2). In our cell culture laboratory, we mainly seeded 300 000 number of MSCs at the beginning of P2 and we mainly harvested approximately 600 000 MSCs at the end of the culture period. One milliliter supernatant of P2 culture medium was collected when the cells were at 70–80% confluency and then frozen at −80°C after filtering through 0.20 μm filter until enzyme-linked immunosorbent assay (ELISA) tests were performed.

BM MSCs were characterized by flow cytometry (Becton Dickinson FACS Aria) using a specific CD
antibody panel (Becton Dickinson and BioLegend) including hematopoietic (CD14, CD34, and CD45) and mesenchymal markers (CD73, CD105, and CD44) (data not shown). The MSCs were further characterized by their capacity to differentiate into adipogenic (Fig. 1B) and osteogenic lineages (data not shown) as previously described. Briefly, adipogenic differentiation was induced in the expanded mesenchymal cell cultures by treatment with 1-methyl-3-isobutylxanthine, dexamethasone, insulin, and indomethacin. Induction was apparent by the accumulation of lipid-rich vacuoles within cells after 21 days that stain with Oil red O. The osteogenic differentiation was promoted in the expanded mesenchymal cell cultures by treatment with dexamethasone, β-glycerol phosphate, and ascorbate in the presence of 10% (v/v) FBS, which caused aggregate or nodule formation. After 21 days, cells stained with Alizarin Red.

**ELISA**

Cytokines levels in BM plasma and MSCs supernatants were measured by ELISA test which was performed using commercially available kits from Raybiotech systems according to the manufacturer’s instructions. The cytokine levels and Ang 1/Ang 2 ratios were measured at diagnosis and after the remission induction therapy of patients and also measured in control group. Also, the cytokine level of conditioning medium which included FBS but no cell was measured. The minimum detectable dose stated by the manufacturer was 30 pg/ml for Ang 1, 10 pg/ml for Ang 2 and 80 pg/ml for SDF 1α.

**Statistical analysis**

All patients (T and B cell ALL) were considered in one group. The cytokine levels and Ang 1/Ang 2 ratios of the patients at diagnosis and after the remission induction therapy were compared with control group statistically. Mean ± SD and/or median (range) values were recorded for numeric variables. Number percent was used for categorical variables. Comparison of two independent groups was tested by Mann–Whitney in numerical variables and chi-square in categorical variables. Comparison of variables before and after treatment was tested by Wilcoxon. Kruskal–Wallis analysis was performed in order to compare the numerical variables according to the risk groups. Spearman rho correlation coefficient was used in order to investigate the relation between the numerical variables. The differences were statistically significant when P value was smaller than 0.05. IBM SPSS Statistics version 21.0 was used for the analysis.

**Results**

Mean age of patients and control groups was 7.4 ± 5.8 and 7.5 ± 4.0 years, respectively. Female/male ratio was 6/14 and 2/6 in patients and control group, respectively. The vast majority of patients were of B lymphocytic lineage. Seven patients were in low risk group, eighth in intermediate and five in high risk group. High risk group existed exclusively of pre B cell ALL patients whereas one patient with T lymphocytic lineage was in high risk group (Table 1). All patients except two achieved remission after remission induction therapy.

At diagnosis (before treatment) BM plasma levels of Ang 1 and SDF 1α were significantly low (P < 0.001 and P = 0.023, respectively) and Ang 2 was high (P < 0.001) when compared to control values (Table 2). Following the remission induction therapy at day +33 levels of Ang 1 and SDF1α increased (P = 0.002 and P = 0.006, respectively) while level of Ang 2 decreased (P = 0.030) when compared to the levels at diagnosis. At the time of diagnosis BM plasma Ang 1/Ang 2 ratio was lower than the control group (P < 0.001) and at day +33 the ratio increased significantly when compared to the levels at diagnosis (P = 0.001).
The pattern of Ang 1 and Ang 2 secretion from P2 MSCs was not different among patients and controls (Table 2). However Ang 1/Ang 2 ratio was significantly lower in supernatants at diagnosis ($P = 0.035$) when compared to control levels, SDF 1α levels did not show a statistically significant change in supernatant samples.

While all patients except two achieved remission after remission induction therapy, statistical analysis to compare the changes in the levels of the angiogenic cytokines with remission status was not available.

Any correlation between the levels of cytokines and the patients’ leukocyte, blast count, hemoglobin level and platelet counts and the blast percentage of the BM were not detected at diagnosis and on day +33.

**Discussion**

Setting up the experimental conditions mimicking human BM microenvironment is a challenging issue due to the complex dynamic cell–cell–matrix–soluble factor interactions and the consistently changing physical conditions *in vitro*. Partial data concerning microenvironment are obtained from co-culture studies. Examination of BM plasma is a simple measure in projecting the *in vivo* BM microenvironment. In present study, some angiopoietic cytokines in BM of patients with ALL were tested in order to assess the role of these cytokines and MSC supernatants were tested in order to understand the contribution of MSCs to the release of these cytokines. It was demonstrated that at diagnosis the levels of Ang 1 and SDF 1α were low while Ang 2 level was high in the BM microenvironment represented by BM plasma levels. After the remission induction phase Ang 1 and SDF 1α levels increased and Ang 2 were found to be lower.

**Table 1** Demographic data of patients and controls are demonstrated. (A) The age and gender of the patients and controls are stated. (B) Differential blood count, BM blast percentage at diagnosis, ALL subtypes, chromosome abnormalities, risk groups and remission status (after induction therapy) of patients are stated. Two patients had t(11q23) which was unfavorable and two had t(12;21) which was favorable.

|                  | Patients                  | Control group             |
|------------------|---------------------------|----------------------------|
| **(A)**          |                           |                            |
| Age              | Mean ± SD                 | 7.4 ± 5.8                  |
| Min-max          |                           | 1.1−17.0                   |
| Gender           | Female                    | 6 (30%)                    |
|                  | Male                      | 14 (70%)                   |
| Total            |                           | 20 (100%)                  |
| **(B)**          |                           |                            |
| Differential blood count | Mean ± SD | 8.7 ± 3.1                  |
| WBC (1/mm³)      |                           | 27 ± 33859                 |
| Absolute blast count (1/mm³) |           | 8919 ± 2328               |
| Platelet (1/mm³) |                           | 66 ± 64706                 |
| BM blast percentage at diagnosis |          | 90.7 ± 9.3                |
| ALL subtypes, n | B cell lineage            | 17                         |
|                  | T cell lineage            | 3                          |
| Chromosome abnormalities, n | Favorable | 2                         |
|                  | Unfavorable               | 2                          |
|                  | No abnormality            | 16                         |
| Risk groups, n   | Low risk                  | 7                          |
|                  | Intermediate risk         | 8                          |
|                  | High risk                 | 5                          |
| Remission status after induction, n | Remission | 18                        |
|                  | Not remission             | 2                          |

A positive correlation between Ang 2 levels in BM plasma and supernatants was detected ($r = 0.520$, $P = 0.010$). Neither Ang 1 nor SDF 1α levels were correlated between BM plasma and MSC supernatants.

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**Table 2** Cytokine levels of patients at time of diagnosis and after induction therapy (on day +33) and control groups. The cytokine levels were measured both in BM plasma and MSCs supernatant.

|                  | BM plasma                  | supernatant                |
|------------------|---------------------------|----------------------------|
| Ang 1 (pg/ml)    | Median (range)            | Median (range)             |
| At diagnosis (n = 20) | 87.09 (2414.59)           | 1371.78 (1502.95)         |
| On day +33 (n = 20) | 2002.40 (11820.57)        | 1018.57 (1179.87)         |
| P value*         | 0.002                     | 0.03                       |
| Control group (n = 8) | 971.29 (1520.52)           | 754.94 (236.5)            |
| P value**        | <0.0001                   | <0.0001                    |
| Ang 2 (pg/ml)    | Median (range)            | Median (range)             |
| At diagnosis (n = 20) | 1371.78 (1502.95)         | 1018.57 (1179.87)         |
| On day +33 (n = 20) | 1371.78 (1502.95)         | 1018.57 (1179.87)         |
| P value*         | 0.002                     | 0.03                       |
| Control group (n = 8) | 754.94 (236.5)            | 754.94 (236.5)            |
| P value**        | <0.0001                   | <0.0001                    |
| Ang 1/Ang 2 ratio |                           | 0.07                       |
| SDF 1α (pg/ml)   | Median (range)            | Median (range)             |
| At diagnosis (n = 20) | 29.50 (37.39)             | 40.91 (67.81)             |
| On day +33 (n = 20) | 29.50 (37.39)             | 40.91 (67.81)             |
| P value*         | 0.023                     | 0.006                      |
| Control group (n = 8) | 41.09 (28.68)             | 41.09 (28.68)             |
| P value**        | 0.023                     | 0.006                      |

*Comparison of patients at time of diagnosis versus on day +33 (after induction therapy).
**Comparison of patients at time of diagnosis versus control group.
decreased. However those soluble factors in MSCs supernatants did not reveal a statistically significant change from control samples. Of interest, a low Ang 1/Ang 2 ratio was also present in MSCs supernatants obtained from BM samples at diagnosis. In order to obtain sufficient number of cells for in vitro studies, MSCs were expanded in culture for several weeks. Therefore, the data obtained from MSCs do not picture in vivo conditions exactly due to the absence of interacting cells and factors in in vitro conditions. Additionally due to the use of fetal calf serum for culture expansion the secretory profile does not represent in vivo levels but give information about the secretory capacity of the cells which were exposed to in vivo leukemic environment when compared with cells obtained from healthy donors. Here we investigated the functional/secretory and dynamic features of MSCs and showed that they do not differ between the time of diagnosis and after induction therapy. Conforti et al. studied the morphological features of MSCs derived from pediatric patients with ALL and demonstrated that MSCs at diagnosis do not differ from those obtained during treatment. However, Mallampati et al. who studied a mouse BCR-ABL+ ALL model observed that tyrosine kinase inhibitors induce MSC-mediated resistance and MSCs may play an essential role in activation of an alternative survival signaling pathway in leukemic cells that protects leukemic cells from chemotherapy.

The effects of Ang 1 and Ang 2 on tumor angiogenesis have been studied in acute myeloid leukemia (AML), chronic lymphocytic leukemia, multiple myeloma, and myelodysplastic syndrome (MDS). To our knowledge this is the first study about the effect of angiopoietins in ALL.

Studies with adult patients with AML usually revealed that Ang 2 is a prognostic factor and high levels of Ang 2 is related with poor outcome. Likewise Hatfield et al. demonstrated that serum Ang 2 levels of 7 untreated adult patients with ALL were higher than control. However Cheng et al. studied the levels of Ang 1, Ang 2, and Tie 2 levels of 208 adult patients with MDS in BM and found that the level of Ang 2 was low whereas Ang 1 and Tie 2 was high. They also confirmed that acute leukemic transformation was more frequent and prognosis was poorer in patients with high Ang 1 levels.

In the present study, Ang 1/Ang 2 ratio of BM plasma was low at the time of diagnosis and increased after the remission induction therapy. Interaction of Ang 1 and Ang 2 and Ang 1/Ang 2 ratio have a critical role in vascular homeostasis in BM stem cell traffic. Furthermore, it is established that Ang 1/Ang 2 ratio is a prognostic factor in solid tumors. However it has not been demonstrated in acute leukemias. Since the number of poor responder subjects was relatively small and the follow-up period was short the correlation of Ang 1/Ang 2 ratio with prognosis could not be evaluated. Hypothetically, the low ratio of Ang 1/Ang 2 at diagnosis is related to leukemic transformation, and increase in this ratio after induction therapy may be attributed to the response to the therapy. Also, hypothetically, the interaction between the leukemic blasts and MSCs might influence Ang 1/Ang 2 ratio. Further studies including co-culture studies are needed to determine the role of Ang 1/Ang 2 ratio in leukemic BM microenvironment. We suggest that the low ratio of Ang 1/Ang 2 at beginning and increase of it after the induction therapy, to some extent might be the nature of pediatric ALL.

Physiologically there is a balance between angiogenic and anti-angiogenic factors. Angiogenic switch defines the disequilibrium resulting in increased/ decreased angiogenesis depending on the dominant side. Hypothetically, the prepotency of Ang 2 at the time of diagnosis leads to an increase in angiogenesis, vascular permeability and peripheral mobilization of the blasts. We suggest that after remission induction therapy with reduction of blasts Ang 1 dominates resulting in stabilization of the newly formed vessels, decrease in vascular permeability and homing of HSCs and residual leukemic stem cells into osteoblastic niche where they remain in quiescent phase. Szmigielska-Kaplon et al. have studied Angs in hematopoietic stem cell mobilization in patients with hematological malignancies and found out that at the time of mobilization levels of Ang 1 was low and Ang 2 was high, when compared with basal levels. Dynamic ratio of Ang 1/Ang 2 might have a role in stem cell (MSCs, endothelial stem cells and HSCs) traffic in BM microenvironment affecting the behavior of leukemic stem cell and might have important roles about angiogenesis, chemotherapy resistance and prognosis in leukemia. Further studies are needed to evaluate these hypotheses.

In literature, SDF 1α has been studied mostly in solid tumors which have more stromal tissue than BM and levels of it were found to be higher than controls. There are a few studies concerning SDF 1α in ALL. Mowafi et al. and Ge et al. showed in the pediatric and adolescent/young adults respectively that serum SDF 1α levels were higher in pediatric patients with ALL at diagnosis and similar to controls. In our study, we measured levels of SDF 1α in BM plasma and demonstrated that patients had lower levels at diagnosis and higher levels after induction therapy, when compared to controls. Van den Bek et al. also demonstrated that CXCL12 levels of 11 pre B cell ALL patients in BM were lower than controls at diagnosis. It is well established that SDF 1α is critical in homing of progenitor and stem cells into BM niches. Lymphoid progenitor
cells express high levels of CXCR4 on surface which makes them a suitable target for SDF 1α. Activation of SDF 1α/CXCR4 leads to adhesion, chemotaxis, proliferation, and survival of leukemic stem cells.\footnote{Iwamoto S, Mihara K, Downing JR, Pui CH, Campana D. Mesenchymal cells regulate the response of acute lymphoblastic leukemia cells to asparaginase. J Clin Invest. 2007;117(4):1049–57.}

Inhibition of this pathway leads to the differentiation and mobilization which makes leukemic cells more sensitive to treatment.\footnote{Tait CR, Jones PF. Angiopoietins in tumours: the angiogenic switch. J Pathol. 2004;204(1):1–10.}

In conclusion, in pediatric ALL at the time of diagnosis high levels of Ang 2 and low levels of Ang 1 and SDF 1α all together may have an effect on opening up the BM niches and promoting the proliferation and peripheral mobilization of blastic population. After the induction therapy low levels of Ang 2 and high levels of Ang 1 and SDF 1α may close down the niches and ends up with homing of leukemic blasts into the niche where they remain in quiescent phase. These results point out that factors related to angiogenesis have important roles in governing stem cell traffic by means of vessel stability. Ang 1, Ang 2, and SDF 1α may have an effect not only on angiogenesis but also homing of leukemic blasts into BM and peripheral mobilization. Taken together, alteration of Ang 1, Ang 2, and SDF 1α expression could be potentially developed as biomarkers for monitoring the effectiveness of chemotherapy. Future experimental studies in co-culture conditions may reveal the direct effect of MSCs to the secretion of angiogenic factors which may have implications in development of targeted therapies.

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