Microenvironmental Remodeling as a Parameter and Prognostic Factor of Heterogeneous Leukemogenesis in Acute Myelogenous Leukemia

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Abstract

Acute myelogenous leukemia (AML) is a heterogeneous disorder characterized by clonal proliferation of stem cell-like blasts in bone marrow (BM); however, their unique cellular interaction within the BM microenvironment and its functional significance remain unclear. Here, we assessed the BM microenvironment of AML patients and demonstrate that the leukemia stem cells induce a change in the transcriptional programming of the normal mesenchymal stromal cells (MSC). The modified leukemic niche alters the expressions of cross-talk molecules (i.e., CXCL12 and JAG1) in MSCs to provide a distinct cross-talk between normal and leukemia cells, selectively suppressing normal primitive hematopoietic cells while supporting leukemogenesis and chemoresistance. Of note, AML patients exhibited distinct heterogeneity in the alteration of mesenchymal stroma in BM. The distinct pattern of stromal changes in leukemic BM at initial diagnosis was associated with a heterogeneous posttreatment clinical course with respect to the maintenance of complete remission for 5 to 8 years and early or late relapse. Thus, remodeling of mesenchymal niche by leukemia cells is an intrinsic self-reinforcing process of leukemogenesis that can be a parameter for the heterogeneity in the clinical course of leukemia and hence serve as a potential prognostic factor. Cancer Res; 75(11); 2222–31. ©2015 AACR.

Introduction

Acute myelogenous leukemia (AML) is a heterogeneous clonal disorder of hematopoietic progenitor cells characterized by excessive proliferation of stem cell-like progenitor cell in bone marrow (BM) with a heterogeneous prognosis (1). Xenotransplantation studies of AML demonstrate a hierarchical organization of AML and identified a subpopulation of leukemia cells called leukemic stem cells (LSC); they arise from the transformation of hematopoietic stem cells (HSC) or reacquisition of self-renewal capability in committed progenitors (2, 3), and can initiate and maintain their leukemic state. As LSCs share stem cell-like properties with normal HSCs, LSCs may be regulated within the BM microenvironment (4).

The BM microenvironment for HSC is characterized by a specialized architecture termed the stem cell niche localized in the perivascular or endosteal area of BM where most HSCs reside (5). Although the niche is comprised of various types of stromal cells from mesenchymal and nonmesenchymal origin (6–8), a subset of mesenchymal stromal cells (MSC) that retain colony-forming potential (CFU-F) and self-renewal ability could reconstitute both types of niches in the heterologous marrow model (9, 10). Subsequent studies identified additional subsets of mesenchymal cells, including early-stage osteoblastic cells expressing Runx2 (11), nestin (12), or leptin-receptor (13), or primitive (Prx-1+1) MSCs (14) as major regulators of HSCs in the BM niche. These niche cells express cross-talk molecules such as JAG1 (15, 16), CXCL12 (14, 17, 18), and ANGPT1 (19) to interact with normal HSCs to regulate their self-renewal (15, 20), quiescence (21–23), and mobilization (24).

The LSCs, when transplanted, engraft in the BM-like normal HSCs, competing for niche with normal HSCs (25) and influence the normal stem cell niche, causing alteration in the microenvironmental regulation. For example, leukemia cells transplanted into mice create an abnormal BM niche to usurp the transplanted normal HSCs into a tumor niche (26). In addition, a transgenic model of chronic myeloid leukemia shows defective homing and retention of HSCs in the niche due to decreased CXCL12 in BM MSCs (27). Similarly, mice with Bcr-Abl–transformed leukemia exhibit analogous alterations of MSCs and osteoblastic cells along with their supporting effects on leukemogenesis (28, 29), thus, giving rise to the concept of the “leukemic niche” (30, 31).

Nevertheless, little is known about the leukemic microenvironment in human leukemic diseases or its clinical relevance to the
leukemogenic process. Here, using a human AML model, we report the leukemic alterations of the microenvironment in AML patient’s BM and their impact on the clinical course of AML patients.

Materials and Methods

Study design and sample collection

For the initial screening of BM stroma in AML, 51 BM samples from treatment-naïve AML patients were randomly collected. For the cohort study on prognosis, another set of BM samples was collected from 48 patients newly diagnosed with AML without prior treatment history who had complete medical records during 5 to 8 years of follow-up. This study was approved by the Institutional Review Boards of St. Mary’s Hospital and Seoul St. Mary’s Hospital. All samples, including umbilical cord blood cells, were obtained after obtaining informed consent.

Analysis of BM stromal cells

The in vitro culture of MSCs and colony-forming assay (CFU-F) were performed as described previously (32). Senescence-associated β-galactosidase (SA-β-gal) activity was examined by a senescence β-galactosidase staining kit (Cell Signaling Technology). Specific subsets of MSCs in fresh BM mononuclear cells were analyzed by flow cytometry after staining with specific antibodies (Supplementary Table S1 for antibodies). Cross-talk molecule expression on MSCs was similarly analyzed by flow cytometry after staining against JAG1 and CXCL12 as described previously (see Supplementary Table S1 for antibodies; refs. 16, 33).

Ex vivo culture of normal hematopoietic and leukemia cells

MSCs were irradiated with 1,500 cGy 24 hours before coculture with normal or leukemic CD34+ cells for 5 days in long-term culture media (HS100, STEMCELL Technologies) in the presence of a cytokine mixture (100 ng/mL human SCF; 100 ng/mL human Flt3L; and 20 ng/mL human IL3, IL6, and G-CSF; ProSpec-Tany TechnoGene Ltd.). For long-term culture-initiating cell (LTC-IC) analysis, CD34+ cells were cocultured with normal MSCs or AML-derived MSCs (AML-MSCs) for 5 days, transferred to a 6-week

Figure 1.
Altered mesenchymal stroma in the BM of AML patients. A, comparison of mesenchymal cell composition in BM. Percentages of primitive MSCs (CD45−31/CD235a−146/CD166+) and differentiated osteoblastic cells (CD45−31/CD235a−146/CD166+) in the total MSC population (CD45−31/CD235a−) in fresh BM (mean ± SEM, n = 51, 11 for AML and normal BM, respectively). B, CFU-F and proliferation of MSCs in AML and normal BM. The percentage of BMs that did not form any CFU-F colonies (no colony), formed colonies, but reached growth arrest within two passages of subculture (growth arrest) and continued proliferating beyond two passages (normal growth) are shown (n = 51 and 11 for AML and normal BM, respectively). C, senescence-associated β-galactosidase activity of AML and normal BM-derived MSCs. Mean ± SEM of three independent experiments (n = 9 and 12 for normal and AML, respectively; ***, P < 0.001). D, CFU-Fs in the BM of normal and age-matched AML patients at initial diagnosis. Box plot shows CFU-Fs obtained from mononuclear cells contained in 1 mL BM with mean ages (n = 11 and 51, for normal and AML, respectively; *, P < 0.05). E, CFU-Fs after AML remission. CFU-Fs in equivalent numbers of BM mononuclear cells (5 × 10^6) were plated to compare normal and hypocellular BM after AML remission (n = 17 and 11 for normal and AML-remission, respectively; *, P > 0.05).
long-term culture, and subjected to a colony-forming assay in semi-solid medium (34).

Animal leukemia transplantation model

BM progenitor cells from C57BL6 mice were transformed to leukemia cells (AML) by transduction of \( \text{MN1} \) as described (35) and transplanted into congenic mice to establish transplantation leukemia model. Repopulating assay in neonatal mice disrupted with bis (Bis-knockout) were performed as described previously (33).

NOD/SCID-\( \gamma \)-null repopulation assay

NOD/SCID-\( \gamma \)-null mice were irradiated with 250 cGy and i.v. injected with hematopoietic cells cocultured on MSCs. Human cell engraftment was analyzed as described previously (36).

Gene expression analysis of MSCs

MSC gene expression was analyzed by Illumina BeadChip (Illumina) array hybridization analysis, using the Linear Models for Microarray Data (LIMMA) method and the R-package for Statistics as described previously (37). For expression profiles, the median absolute deviation was calculated, and highly variable genes were selected for hierarchical clustering with average linkage. Gene set enrichment analysis was performed using the Gene Ontology (GO) categories from MSigDB (PMID: 16199517).

Statistical analysis

For gene set enrichment analysis, normal \( P \) values of significance were estimated for 1,000 permutations of genes for each GO category to select significant GO categories (i.e., \( \text{FDR} < 0.1 \)). The predictive performance of each stromal cell parameter for leukemic relapse was determined by receiver operator characteristic (ROC) curve analysis and by calculating the AUCs with standard errors. All data were analyzed using SAS version 9.3 (SAS Institute Inc.), and the level of significance was set at a \( P \) value of <0.05.

Results

Altered mesenchymal cells in BM in AML

To investigate the characteristics of the BM microenvironment specific to human leukemic conditions, we first analyzed the mesenchymal cellular composition of the fresh BM stromal cells in treatment-naïve patients initially diagnosed with AML. Compared with normal BM, the frequency of primitive subsets (CD146\(^+\)166\(^-\)) of MSCs (10, 38, 39) was significantly lower, whereas the frequency of mature osteoblastic cells (CD146\(^-\)166\(^+\); ref. 40) was higher (Fig. 1A), indicating an overall shift in mesenchymal differentiation. We subsequently examined the colony formation of MSCs in normal and leukemic BM, because niche cells are enriched in colony-forming mesenchymal progenitors (9, 10). Compared with normal BM, BM mononuclear cells from AML patients frequently failed to form colonies, and exhibited a higher frequency of accelerated growth arrest (within two passages of subculture) and higher senescence-associated \( \beta \)-galactosidase activity (Fig. 1B and C and Supplementary Fig. S1A). Even among AML-MSCs that continued to grow beyond three passages, decreased proliferation was observed during the 60-day subculture (Supplementary Fig. S1B). Altered MSC function was observed in AML patients irrespective of AML subtype (Supplementary Fig. S1A). Of note, although the overall numbers of CFU-Fs were lower in AML BM than age-matched normal BM, no difference was observed when AML patients who achieved...
complete remission (CR; Fig. 1D and E), indicating that mesenchymal alterations reflect ongoing leukemogenic activities in AML BM.

To determine whether these mesenchymal alterations can be caused by leukemic blasts, we examined MSCs cocultured with leukemic blasts and compared their transcription profiles with those of MSCs cocultured with normal hematopoietic progenitors (Fig. 2A). Hierarchical clustering of highly variable genes clearly segregated the MSCs cocultured with normal and leukemic blasts (Fig. 2B), indicating a substantial difference in transcriptomes. Gene set enrichment analysis was performed to identify the candidate molecular functions associated with these transcriptomic changes. Overall, 11 and 80 GO categories exhibited significant enrichment with upregulated and downregulated genes, respectively, in MSCs cocultured with leukemic CD34+ cells compared with those cocultured with normal CD34+ cells (Fig. 2C; Supplementary Table S3). Of note, among GO categories downregulated under leukemic conditions, genes for "cell-cycle" and related functions (e.g., "chromosome" and "DNA replication") were significantly enriched, consistent with the loss of proliferation in AML-MSCs. The enrichment plot of 105 genes from "cell-cycle"–related gene sets is shown with the top 20 leading-edge genes (Fig. 2D; Supplementary Table S3). In contrast, among the GO categories upregulated in leukemia-cultured MSCs, two cytokine-related GO functions were observed: "chemokine receptor binding" and "chemokine activity" (Fig. 2C and Supplementary Fig. S2). These results indicate leukemia cells can indeed induce transcriptomic reprogramming of MSCs distinct from normal hematopoietic cells with prominent suppression of cell-cycle–related genes and upregulation of cytokine-related genes.

The leukemic niche resets microenvironmental cross-talk for normal and leukemia cells

Leukemia-induced mesenchymal alterations were further examined with respect to changes in the expressions of major cross-talk molecules, JAG1 and CXCL12, which regulate HSCs in the BM niche (5, 15, 16). First, the mesenchymal expression of JAG1, was examined during coculture with normal or leukemia cells (Fig. 3A). Coculture with normal hematopoietic progenitors increased and sharply decreased the percentage of JAG1+ cells in normal MSCs and AML-MSCs, respectively, demonstrating distinct responses in these MSCs (Fig. 3B). Accordingly, normal hematopoietic
progenitors cocultured with AML-MSCs exhibited significantly suppressed downstream notch signals HES1 and HES5 compared with coculture with normal MSCs (Fig. 3C). In contrast, coculture with leukemic blasts did not alter the percentages of JAG1⁺ cells in normal MSCs or AML-MSCs (Fig. 3D). These results indicate that JAG1 cross-talk is selectively suppressed for normal hematopoietic progenitors, but maintained for leukemic blasts on AML-MSCs. Likewise, the percentage of CXCL12⁺ cells among cocultured MSCs increased markedly and selectively in response to leukemia cells, but decreased during coculture with normal hematopoietic cells (Fig. 3E).

Moreover, the fresh uncultured BM stromal cells from AML patients exhibited higher percentage of CXCL12⁺ MSCs than in normal BM, indicating similar alterations of cross-talk under in vivo condition (Fig. 3F and Supplementary Fig. S3). These results indicate that the microenvironment altered by leukemia cells differentially responds to normal and leukemia cells to reset the cross-talks among normal and leukemia cells.

Altered microenvironments provide a selective advantage to leukemia cells over normal HSCs

Next, we investigated the functional impacts of leukemic MSCs on normal hematopoietic function by coculturing normal CD34⁺ cells with normal MSCs or AML-MSCs (Figs. 4A and 5A). Significant ex vivo expansion of CD34⁺ cells was observed in coculture with normal MSCs but not with AML-MSCs (Fig. 4B). Similarly, when transplanted into NOD/SCID-γnull mice (41), normal hematopoietic progenitors cocultured on AML-MSCs exhibited lower repopulating activities than cells cocultured with normal MSCs (Fig. 4C). In particular, analysis of HSCs by LTC-IC assay (42) showed a profound loss of LTC-ICs in CD34⁺ cells cocultured with AML-MSCs, sharply contrasting with their maintenance with normal MSCs (Fig. 4D). These results demonstrate AML-MSCs suppress normal hematopoiesis and that primitive hematopoietic cells are most profoundly affected by the deteriorating effects of leukemic MSCs.

In contrast, when various types of leukemia cells were cocultured with normal MSCs or AML-MSCs, there were no differences in proliferation in vitro (Fig. 5B). Similarly, there was no difference in leukemogenesis in vivo between NOD/SCID-γnull mice transplanted with leukemia cells cocultured with normal MSCs or AML-MSCs (Fig. 5C and Supplementary Fig. S4), indicating resistance of leukemia cells to the detrimental niche. Moreover, coculture with AML-MSCs drove greater proportions of leukemia cells into quiescence and conferred greater resistance to Ara-C–induced apoptosis than coculture with normal MSCs (Fig. 5D and E).

These results collectively demonstrate that the leukemic microenvironment distinctively affects normal and leukemia cells, selectively suppressing normal hematopoietic cells, but...
supporting the leukemogenic activity and chemoresistance of leukemia cells.

Animal leukemia transplantation model

Given the characteristics of the leukemic niche in human AML, we determined whether they can be reproduced in an animal model engrafted with leukemia cells. Thus, we established a mouse leukemia model for AML by transplanting MN1-induced leukemia (Fig. 6A; ref. 43). Examination of the BM microenvironment showed a profound loss of MSCs and decreased self-renewal of mesenchymal progenitor cells to produce secondary CFU-Fs (Fig. 6B and C). We subsequently investigated the engraftment advantage of leukemia cells over normal hematopoietic cells in deteriorated mesenchymal niche. To this end, we used a Bis−knockout mouse model, where the mesenchymal niche of BM was deteriorated causing a microenvironmental defect in their hematopoiesis (Fig. 6D; ref. 33). When normal hematopoietic or leukemia cells (MN1) were transplanted into Bis−knockout mice, normal hematopoietic cell engraftment was lower in Bis−knockout mice than wild-type mice (Fig. 6E). However, leukemia cells exhibited comparable engraftment in Bis−knockout and wild-type mice, indicating that the leukemogenic process is more resistant to the deterioration of the mesenchymal niche (Fig. 6F). These results collectively indicate that the alteration of the mesenchymal niche by leukemia cells is an intrinsic process of leukemogenesis and that leukemia cells can have a selective advantage in this altered microenvironment.

Stromal remodeling as a factor in the heterogeneous clinical course of leukemia patients

Given that leukemic stromal remodeling functionally influences normal hematopoietic and leukemogenic activities, we hypothesized that differences in the pattern of stromal alteration are an underlying factor of the heterogeneity of the clinical course of AML patients. Accordingly, we examined the association between the BM stromal changes at initial AML diagnosis and subsequent clinical course for 5 to 8 years after remission. Stromal cell composition in BM biopsy taken at initial diagnosis was examined in AML patients who maintained CR for 5 to 8 years, relapsed after remission, and exhibited a refractory response to chemotherapy along with BM from normal donors (see Supplementary Table S4 for patients).

Although individual AML patients exhibited heterogeneity in stromal cell composition, there were substantial differences in the BM content of mesenchymal cellular components, but not endothelial cells, among the AML patients who maintained CR and those relapsed after remission; the BM of the relapse group exhibited more MSCs, osteoblastic cells (OB), CFU-Fs, and primitive MSCs (P-MSC; CD146+/CD45−/CD31−/CD235a−/CD166+), a subpopulation of MSCs enriched with nestin+ cells (Supplementary Fig. S5), than the CR group (Supplementary Fig. S6A and S6B).

Therefore, to determine whether such differences in BM stromal cell patterns at the initial diagnosis of AML can identify patients with a high risk of relapse, we performed ROC curve analysis of...
niche cells (27, 29, 44). Similarly, changes in OBs or MSC numbers in leukemia models reported various types of changes in the BM cells and the BM microenvironment. Recently, studies using ani-

Discussion

became stronger when early (<1 year) and late (>1 year) relapse were analyzed separately; early relapse (<1 year) was associated with greater P-MSC content in BM and with significant predictability (AUC, 0.8 ± 0.08), which further strengthened for relapse within 6 months after remission (AUC, 0.88 ± 0.06; Fig. 7B). In contrast, patients with late relapse exhibited significantly more MSCs or OBs but not P-MSCs in BM than in those with CR, with high predictabilities for late relapse (AUC, 0.91 ± 0.06 and 0.88 ± 0.08 for MSCs and OBs, respectively; Fig. 7B). These results indicate that early and late relapse of AML are associated with distinct stromal microenvironments, that is, large numbers of P-MSCs in BM are strongly associated with early relapse, whereas large numbers of MSCs or OBs are significantly associated with late relapse.

These findings collectively suggest that differences in stromal patterns in leukemic BM at initial diagnosis are associated with different clinical courses in AML patients (Fig. 7C).

Howe
ty, systemic analysis of the microenvironmental alterations in various leukemic conditions have not been made, nor their relevance to clinical courses. Our initial analysis of samples from AML patients revealed a significant loss of primitive MSC populations, which is associated with loss of MSC colonization and self-renewal. In addition, although normal HSCs was shown to induce transcriptional changes in MSCs (47), leukemic blasts reprogram the transcriptomes of MSCs distinct from normal hematopoietic cells, profoundly downregulating cell-cycle-promoting genes and upregulating cytokine-related genes. The altered MSCs also reset niche cross-talks by distinctively altering expression of cross-talk molecules such as JAG1 and CXCL12 for normal and leukemia cells. Accordingly, leukemic MSCs selectively suppress normal hematopoietic functions while sparing the leukemogenic activity and chemoresistance of leukemia cells, thereby contributing to the clonal dominance of leukemia cells over their normal hematopoietic counterparts in the BM. In particular, LTC-ICs, the primitive hematopoietic subpopulation analogous to in vivo long-term repopulating cells (48), were most profoundly lost during co-culture with AML-MSCs; in contrast, their repopulation is less severely affected in NOD/SCID-γ null mice, into which both long- and short-term hematopoietic progenitors can be engrafted (49). Thus, primitive hematopoietic progenitors appear to be more vulnerable to the deteriorating effects of leukemic MSCs. Corroborating this notion, AML is frequently associated with a loss of multilineage hematopoiesis, including anemia, as well as a loss of leukocytes or platelets as a major cause of morbidity. Hence, the leukemia-induced remodeling of the niche resulting in a deteriorated microenvironment can be viewed as another pathogenic mechanism of leukemic diseases.

Interestingly, the alteration of the mesenchymal niche was reproduced in the transplantation leukemia model in mice, which

Figure 6. Alteration of mesenchymal niche in the animal transplantation leukemia model and effects on leukemogenesis. A, schematic of the animal transplantation leukemia model established by transplanting MN1-induced AML cells. Representative flow-cytometry plots for leukemic engraftment and BM section are shown. B, total number of MSCs (CD45−; Ter119−) in BMs of mice transplanted with media (HF2), normal BM cells, or AML cells (MN1; three experiments, n = 8; *P < 0.05). C, self-renewal of mesenchymal progenitors determined by the number of secondary CFU-Fs from each primary CFU-F (three experiments, n = 8).

D, schematic of experimental design to compare the influence of defective mesenchymal niche on the engraftment of normal and leukemia cells. E, numbers of normal hematopoietic cells engrafted in each neonatal wild-type or Bis knockout (KO) mice (five experiments, n = 12). F, numbers of MN1-AML cells engrafted in neonatal wild-type or Bis-knockout mice (five experiments, n = 10).
exhibited a marked loss of mesenchymal stroma and self-renewal of mesenchymal progenitors. Furthermore, the advantage of leukemia cells over normal hematopoietic cells under such a deteriorated microenvironment was also observed in the mouse model with defective mesenchymal niche in BM, the Bis-knockout model. Thus, the leukemia-induced remodeling of the mesenchymal niche, which distinctly influences normal and leukemia cells, appears to be an intrinsic pathogenic process of leukemogenesis in both animal and clinical models.

Of note, although leukemia cells induce various patterns of stromal alterations in BM, the mechanisms of various stromal changes in AML patients remain unclear. Recent animal studies demonstrate that excessive cytokine secretion, which also differs with respect to specific oncogenic mechanisms, is a force driving mesenchymal niche alterations (27–29). The present study revealed that the genes upregulated in leukemia-cultured MSCs were highly enriched among GO categories related to “chemokine activity” implicating similar involvement of excessive cytokine activity in this process. However, studies characterizing the mechanisms underlying heterogeneity in stromal remodeling in each AML patient as well as expression studies under in vivo conditions to overcome potential discrepancies from in vitro conditions are warranted.

Nevertheless, the present study demonstrates the clinical significance of stromal remodeling as an intrinsic process of self-reinforcing leukemogenesis and factor underlying the heterogeneous clinical course in AML patients; thus, the difference in stromal cell components in BM at initial diagnosis is closely associated with the clinical course, particularly the maintenance of CR for 5 to 8 years or relapse after remission during follow-up. The reason for the observed differences in leukemogenesis with respect to stromal environment is unclear.
However, recent studies demonstrate heterogeneous subtypes of MSCs in BM exhibit different niche function and that the primitive subsets of MSCs play a dominant role in the maintenance and self-renewal of HSCs (14). Therefore, heterogeneity in subpopulations of MSCs in BM may provide different niche environments for leukemia stem cells, leading to heterogeneous kinetics of leukemia relapse in each distinct stromal environment. In this case, it is plausible that a lack of P-MSCs in BM would preclude the maintenance and self-renewal of the LSCs responsible for relapse, leading to the maintenance of CR; meanwhile, high levels of P-MSCs or MSCs/osteoclastic cells would support the maintenance and/or self-renewal of LSCs in a distinct manner (Fig. 7C).

Thus, the present findings indicate the patterns of BM stromal changes at initial diagnosis of AML may predict patients with a high risk of relapse. However, although our study provides insights regarding the clinical relevance of stromal changes for leukemogenesis as a hypothesis-generating study, its application as a clinical biomarker requires additional cohort studies with larger sample sizes as a “hypothesis-testing” study.

In conclusion, the present study demonstrates that the leukemia-induced remodeling of the BM microenvironment is an intrinsic part of leukemogenesis and may be responsible for the heterogeneity of the clinical course of AML, and hence a potential prognostic parameter in AML.

References

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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