MIF-Induced Stromal PKCβ/IL8 Is Essential in Human Acute Myeloid Leukemia

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Abstract

Acute myeloid leukemia (AML) cells exhibit a high level of spontaneous apoptosis when cultured in vitro but have a prolonged survival time in vivo, indicating that tissue microenvironment plays a critical role in promoting AML cell survival. In vitro studies have shown that bone marrow mesenchymal stromal cells (BM-MSC) protect AML blasts from spontaneous and chemotherapy-induced apoptosis. Here, we report a novel interaction between AML blasts and BM-MSCs, which benefits AML proliferation and survival. We initially examined the cytokine profile in cultured human AML compared with AML cultured with BM-MSCs and found that macrophage migration inhibitory factor (MIF) was highly expressed by primary AML, and that IL8 was increased in AML/BM-MSC cocultures. Recombinant MIF increased IL8 expression in BM-MSCs via its receptor CD74. Moreover, the MIF inhibitor ISO-1 inhibited AML-induced IL8 expression by BM-MSCs as well as BM-MSC–induced AML survival. Protein kinase C β (PKCβ) regulated MIF-induced IL8 in BM-MSCs. Finally, targeted IL8 shRNA inhibited BM-MSC–induced AML survival. These results describe a novel, bidirectional, prosurvival mechanism between AML blasts and BM-MSCs. Furthermore, they provide biologic rationale for therapeutic strategies in AML targeting the microenvironment, specifically MIF and IL8.

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Introduction

Survival of patients with acute myeloid leukemia (AML) is presently poor; two thirds of young adults and 90% of older adults die of their disease (1). Even in patients who achieve remission with chemotherapy, relapse is common and occurs from minimal residual disease sequestered in protective niches in the bone marrow microenvironment (2). Accordingly, it is envisaged that improved outcomes will come from novel treatment strategies derived from an improved understanding of the biology of AML within the bone marrow microenvironment.

AML cells exhibit a high level of spontaneous apoptosis when cultured in vitro but have a prolonged survival time in vivo, indicating that the tissue microenvironment plays a critical role in promoting AML cell survival (3–6). Knowledge of the complexity of the bone marrow microenvironment is increasing, especially with respect to the bone marrow mesenchymal stromal cells (BM-MSC), which are considered a major protective cell type (7). BM-MSCs generate various factors whose primary functions are to influence tumor cell survival and homing (4, 8, 9). The apoptotic defect in AML is not cell autonomous but highly dependent on extrinsic signals derived from their microenvironment. The complex cell–cell interactions between the AML tumor cells and their microenvironment are therefore essential for tumor growth and survival and thus present an attractive target for novel drug therapies.

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine, which under normal conditions regulates cell-mediated immunity and inflammation (10). In cancer, MIF is overexpressed in a number of solid tumors, including breast, prostate, and colon cancers (11–13). MIF has also been shown to be overexpressed in various blood cancers, including chronic lymphocytic leukemia (CLL; ref. 14). In CLL, MIF is expressed by the malignant cells and induces protective IL8 release in an autocrine-dependent manner. Blocking either MIF or IL8 reduces survival of CLL. The increased secretion of IL8 from tumor cells is thought to have wider significance to the tumor microenvironment. Serum IL8 is known to be higher in patients with AML, myelodysplasia (MDS), and non-Hodgkin lymphoma than in normal controls, and levels of IL8 in these patients are similar to those found in patients with multiple organ failure of nonseptic origin (15, 16). Furthermore, leukemic blasts from the majority of patients with AML constitutively express IL8 (17). In addition, inhibition of the IL8 receptor, CXCR2, selectively inhibits proliferation of MDS/AML cell lines and patient samples (18). Together, these studies suggest that MIF and IL8 are functionally important in regulating the survival and proliferation of multiple tumors, including AML.

In the current study, we investigate how AML cells program BM-MSCs via MIF to produce the survival cytokine IL8 and
characterize the signaling pathways underlying this interdependent cell–cell communication.

Materials and Methods

Materials

Anti-PKC, MAPK, and AKT antibodies were purchased from Cell Signaling Technology. Anti-CD74, anti-CXCR2, and anti-CXCR4 antibodies were purchased from Miltenyi Biotec. All inhibitors were purchased from Tocris. The CD74 blocking antibody was purchased from BD Biosciences. Proteome Profiler Human XL array and recombinant human MIF were purchased from R&D Systems. MIF ELISA was purchased from BioLegend. IL8 ELISA was purchased from eBioscience. All other reagents were obtained from Sigma-Aldrich.

Cell culture

For primary cell isolation, heparinized blood was collected from volunteers, and human peripheral blood mononuclear cells were isolated by Histopaque (Sigma-Aldrich) density gradient centrifugation. AML samples that comprised less than 80% blasts were purified using the CD34 Positive Selection Kit. Cell type was confirmed by microscopy and flow cytometry. BM-MSCs were isolated by bone marrow aspirates from AML patients. Mononuclear cells were collected by gradient centrifugation and plated in growth medium containing DME and 20% FBS and 1% L-glutamine. The nonadherent cells were removed after 2 days. When 60% to 80% confluent, adherent cells were trypsinized and expanded for 3 to 5 weeks. BM-MSCs were checked for positive expression of CD105, CD73, and CD90 (BM-MSC markers) and the lack of expression of CD45 and CD34 by flow cytometry. BM-MSCs support AML survival and proliferation (4, 5, 19). To study the cell–cell communication between BM-MSCs and AML cells, we established a coculture system using primary AML cells and BM-MSCs derived from treatment-naïve AML patients. Here, we show a significant difference in primary AML survival when cultured on BM-MSCs for 6 and 14 days compared with AML blast survival when cultured in basal media alone (Fig. 1A; Supplementary Fig. S1). Supplementary Figure S2 shows the different combinations of AML and BM-MSCs in all experiments.

Results

BM-MSCs support AML survival

The microenvironment supports AML survival and proliferation (4, 5, 19). To study the cell–cell communication between BM-MSCs and AML cells, we established a coculture system using primary AML cells and BM-MSCs derived from treatment-naïve AML patients. Here, we show a significant difference in primary AML survival when cultured on BM-MSCs for 6 and 14 days compared with AML blast survival when cultured in basal media alone (Fig. 1A; Supplementary Fig. S1). Supplementary Figure S2 shows the different combinations of AML and BM-MSCs in all experiments.

To determine what factors are responsible for improved primary AML blast survival on BM-MSCs, we analyzed the profile of cytokines and chemokines present in primary AML cultures, BM-MSC cultures, and primary AML blasts cultured in combination with BM-MSCs. Cytokine array profiles of the three culture conditions (Fig. 1B) show a consistent upregulation of IL8 in the coculture sample media (Fig. 1C). Moreover, we also observed high levels of MIF in all AML supernatants, low levels of MIF in all BM-MSC supernatants, and high levels of MIF in the AML/BM-MSC cocultures (Fig. 1B and D). To verify these observations, we carried out IL8- and MIF-specific ELISAs. IL8 concentrations peak at 8 and 24 hours in the AML/BM-MSC coculture supernatants (Fig. 1E), whereas MIF concentrations were high and at similar levels in AML culture supernatants and AML/BM-MSC coculture supernatants (Fig. 1F).
AML-derived MIF induces IL8 expression in BM-MSCs

Next, we looked to determine whether BM-MSCs needed direct contact with AML to increase IL8 expression. RT-PCR showed that IL8 mRNA from BM-MSCs incubated with AML increased by 57-fold when in direct contact and by 50-fold when in indirect contact with AML blasts (Fig. 2A). This confirms that direct tumor cell to stromal cell contact is not necessary for AML to induce increased IL8 expression by BM-MSCs.

Next, we examined the mRNA expression levels of MIF in primary AML (n = 5) and BM-MSC (n = 5) cultures. RT-PCR showed that primary AML cultures, but not BM-MSC cultures, express high levels of MIF mRNA under normal basal conditions (Fig. 2B). As MIF expression has been shown to be increased in AML patients compared with normal patients (20) and the ability of MIF to induce IL8 production by primary CLL (14), we hypothesized that MIF from AML was responsible for the increased IL8 expression in BM-MSCs. To test this hypothesis, we stimulated BM-MSCs with 100ng/mL recombinant human MIF and assayed for IL8 mRNA and protein expression over a period of 24 hours. We show that IL8 mRNA and protein increased (Fig. 2C and D) in response to MIF. To confirm that MIF secreted from AML cells regulates IL8 expression, we used ISO-1, a nontoxic inhibitor of MIF, which functions by binding to bioactive MIF at its N-terminal tautomerase site (21). MIF-stimulated BM-MSCs pretreated with ISO-1 showed a decrease in IL8 mRNA levels compared with untreated BM-MSCs (Fig. 2E). Moreover, AML survival was inhibited when cultured with BM-MSCs in the presence of ISO-1 compared with control AML–BM-MSC cultures (Fig. 2F). Together, these data confirm that MIF secreted by AML cells induces IL8 expression in BM-MSCs.

MIF-induced IL8 upregulation is mediated through CD74

Depending on the cellular context and the disease involved, MIF signaling is mediated by its receptors CXCR2 (IL8 receptor, ILR8) and/or CXCR4 (stromal-derived factor 1 receptor), and/or CD74 (22, 23). BM-MSCs have been reported to express all three receptors (24–26). Using CD105 as a BM-MSC marker to confirm mesenchymal cell phenotype, we show that CD74 and CXCR4 are expressed but CXCR2 is not expressed on
all primary BM-MSCs isolated from AML patients (Fig. 3A). BM-MSCs were further characterized using CD73 and CD90, and lack of CD45 expression.

We used specific inhibitors of CXCR2, CXCR4, and CD74 to determine which receptor/s were responsible for MIF-induced IL8 upregulation. Inhibition of CXCR2 and CXCR4 using pertussis toxin (a GPCR inhibitor) had no effect on MIF-induced IL8 mRNA expression (Fig. 3B). However, the anti-

CD74 blocking antibody inhibited MIF-induced IL8 expression in BM-MSCs (Fig. 3C). These results suggest that CD74 is the dominant receptor in regulating MIF-induced IL8 expression in AML patient-derived BM-MSCs. To further characterize this interaction, we used lentiviral-mediated knockdown (KD) of CD74 in AML patient-derived BM-MSCs, confirming reduced mRNA and protein expression of CD74 after transduction with control KD or CD74 KD lentivirus (Fig. 3D). Furthermore, we
demonstrate that CD74 knockdown inhibits MIF-induced IL8 mRNA expression in AML patient-derived BM-MSCs (Fig. 3E).

Pharmacologic inhibition of PKCβ inhibits MIF-induced IL8 induction in BM-MSCs

We next investigated the signaling cascade in AML patient-derived BM-MSCs downstream of MIF-induced CD74 activation. It has been shown that MIF binding to CD74 activates downstream signaling through the PI3K/protein kinase B (AKT) and MAPK signaling pathways and promotes cell proliferation and survival (27). In addition, Lutzny and colleagues recently described the activation of a PKC pathway in murine stromal cells cocultured with chronic lymphocytic leukemia (28). We treated AML-stimulated BM-MSCs with LY294002 (a PI3K/AKT inhibitor), PD98059 (a MAPK kinase (MEK) 1 inhibitor), or Ro-31-8220 (a PKC pan inhibitor) to determine which pathway/s regulate AML-induced BM-MSC IL8 mRNA induction. We show that Ro-31-8220, the PKC inhibitor, was able to significantly inhibit IL8 expression by approximately 80%, whereas LY294002 and PD98059 had little or no effect (Fig. 4A). Similarly, we found that Ro-31-8220 was able to inhibit IL8 expression by circa 90% in experiments where BM-MSCs were directly activated using recombinant human MIF (rhMIF; rather than AML cells; Fig. 4B). However, in addition, we observed that PD98059 was able to moderately inhibit rhMIF-induced IL8 mRNA induction in BM-MSCs by approximately 30% (Fig. 4B).

To clarify whether PKC, MAPK, or both are activated in response to MIF, we performed Western blot analysis on BM-MSCs for specific phosphorylation of PKC isoforms, MAPK, or AKT in response to MIF activation. BM-MSCs from 4 patient samples treated with MIF had no increase in phosphorylation of AKT and MAPK (Fig. 4D). Next, the PKC isoform–specific inhibitors, Go6976 (PKCα/βII) and PKCβ (29), were used to block MIF-induced IL8 expression in BM-MSCs. Both inhibitors showed inhibition of MIF-induced IL8 upregulation (Fig. 4E). Finally, we used lentiviral-mediated KD for PKCβ, confirming reduced mRNA expression of PKCβ after transduction of BM-MSCs with control KD or PKCβ KD virus (Fig. 4F).
demonstrate that knockdown of PKCβ inhibits MIF-induced IL8 mRNA expression (Fig. 4G). Together, these results confirm that MIF-induced IL8 expression in AML patient-derived BM-MSCs requires PKCβ.

Targeting the MIF–PKCβ–IL8 axis disrupts BM-MSC–induced protection of primary human AML blasts

Finally, to examine the effect of blocking IL8 on BM-MSC protection and survival of primary AML blasts, we cocultured
primary AML blasts derived from treatment-naïve AML patients with BM-MSCs (either control KD or IL8 KD). First, we used lentiviral-mediated KD for IL8. Figure 5A and B shows the mRNA expression and protein expression of IL8 after transduction of BM-MSCs with control KD or IL8 KD virus. Figure 5C demonstrates that knockdown of IL8 inhibits MIF-induced IL8 mRNA expression. Next, we show that KD of IL8 in BM-MSCs significantly inhibits AML survival when in coculture compared with control KD BM-MSCs (Fig. 5D). Finally, blocking the IL8R using SB225002 inhibited AML survival when cultured with BM-MSCs (Fig. 5E). Taken together, these results identify a novel protumoral regulatory pathway in the AML microenvironment.

Discussion

AML is primarily a disease of the elderly with a median age at diagnosis in the Swedish Acute Leukemia Registry of 72 years (29). Outcomes for the 75% of patients who get AML over the age of 60 remain generally poor, largely because the intensity and side effects of existing curative therapeutic strategies (which are commonly used to treat younger fitter patients), coupled with patient comorbidities, frequently limit their use in this older less fit population (30). Accordingly, there is an urgent need to identify pharmacologic strategies to tackle AML, which are not only effective but can also be tolerated by both older and less well patients. It is envisaged that treatments that target the tumor microenvironment may well help realize this goal.

Here, we report a novel survival pathway within the human AML microenvironment, which functions as a feedback/autocrine loop involving the constitutive expression of the chemokine MIF by the AML blasts, which in turn induces IL8 expression in BM-MSCs. Interestingly, another group showed that the repertoire of constitutive in vitro chemokine release from AML shows variation between different AML patient samples (31). We find that although baseline expression of MIF by AML varies between patient samples tested, all samples analyzed expressed MIF. Moreover in coculture experiments, AML patient-derived BM-MSCs were found to be ubiquitously responsive to AML-derived MIF, which resulted in an increase in IL8 expression by the BM-MSCs. This is in keeping with similar reports on other cytokine pathways, which have shown that BM-MSCs can constitutively express various chemokines (32), and AML cells are able to respond to these chemokines (32, 33). In this study, we also examined the genotype of six BM-MSCs used for the experiments and found three of six to be normal and in other three, the genotyping failed (Supplementary Table S2). This is apparently in contrast to Huang and colleagues, who found that three of four BM-MSCs from AML patients tested had cytogenetic abnormalities within the stromal cells (34). Presently therefore, the incidence and functional consequences of cytogenetic mutations within stromal cells remain undefined. Nevertheless, taken together, despite the established heterogeneity in AML, we find the MIF/IL8 autocrine loop a constant and functional consequence of cytogenetic abnormalities within the stromal cells. The Mann-Whitney U test was used to compare between treatment groups (*, P < 0.05).

Figure 5.
Targeting MIF–PKC–IL8 axis in AML disrupts BM-MSC–derived protection. A and B, BM-MSCs from six samples were infected with control shRNA or IL8 shRNA for 72 hours and analyzed for IL8 mRNA expression by RT-PCR and protein expression by ELISA. C, BM-MSCs from six samples were infected with control shRNA or IL8 shRNA for 72 hours and then treated with recombinant MIF and analyzed for IL8 mRNA expression by RT-PCR.
D, BM-MSCs were infected with control shRNA or IL8 shRNA for 72 hours and cocultured with AML blasts from seven samples for 48 hours. AML blast number was assessed using Trypan blue exclusion hemocytometer–based counts (n = 7).
E, BM-MSCs were pretreated with SB225002 (100 nmol/L) for 30 minutes before the addition of primary AML blasts from 10 samples for 48 hours. AML blast number was assessed using Trypan blue exclusion hemocytometer–based counts. The Mann-Whitney U test was used to compare between treatment groups (*, P < 0.05).
colleagues have reported that inhibition of the IL8 receptor CXCR2 selectively inhibits immature hematopoietic stem cells from MDS/AML samples (17, 18). In other malignancies, IL8 has been characterized in endothelial cells and tumor-associated macrophages, suggesting that IL8 has a function in the liver and prostate tumor microenvironments (38, 39). As rodents lack a direct homologue of IL8, we purified BM-MSCs extracted from patient bone marrow aspirates at the time of diagnosis of AML. Our study describes for the first time how AML stimulates the production of IL8 from BM-MSCs and inhibiting this process prevents AML survival.

Extensive studies of MIF function have revealed its central role in innate and adaptive immunity (10). More recently, the ability of this cytokine to support tumor progression has been highlighted, revealing MIF as a potential target for anticancer therapies in melanoma and colon cancer (40). MIF occurs in immunologically distinct conformational isoforms, reduced MIF and oxidized MIF (oxMIF), with the latter predominantly expressed in patients with inflammatory diseases (41) and is highly expressed by various cancer cell lines (42). This has led to the evaluation of an oxMIF = blocking antibody (imalumab) in early-phase clinical studies of cancer cell lines (42). This has led to the evaluation of an oxMIF = blocking antibody (imalumab) in early-phase clinical studies of selected solid tumors (https://clinicaltrials.gov/ct2/show/NCT01765790). Our findings provide a biological rationale for the clinical assessment of imalumab or other MIF inhibitors in AML patients.

Activation of PKC signaling pathway has been characterized in cancer cells. In hematologic malignancies, different PKC isoforms have been identified as key players in the leukemia microenvironment. In multiple myeloma, pharmacologic inhibition of activated PKCβII using enzastaurin inhibited growth factors and cytokines secreted by multiple myeloma–derived bone marrow stromal cells (28). In CLL, PKCβ is immediately downstream of the B-cell receptor and has been shown to be important to CLL cell autocrine survival and proliferation in vitro (43). PKCβ is also essential for the development of CLL in the TCL1 transgenic mouse model, making it a valid therapeutic target in this malignancy (44). Furthermore, induction of PKCβII in stromal cells is required for the survival of leukemic B cells, and stromal PKCβII is upregulated in samples from CLL, ALL, and MCL patients (28). Our results demonstrate that in primary samples from AML patients at diagnosis, PKCβ is phosphorylated in the BM-MSCs in response to MIF stimulation. This leads us to hypothesize that this pathway may commonly be activated in other hematologic malignancies, and moreover, the cancer cell is inducing this activation. In summary, this study links secretion of MIF from primary AML to a specific BM-MSC pathway, utilizing PKCβ to feedback survival signals, including IL8 secretion to AML. In doing so, we have identified in vitro the potential efficacy of targeting any one of these molecules to disrupt AML/BM-MSC prosurvival interactions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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