

Original Article

Molecular biological characteristics of the recruitment of hematopoietic stem cells from bone marrow niche in chronic myeloid leukemia

Biao Zhu, Jianbo Zhang, Jiao Chen, Chenglong Li, Xiaodong Wang

Department of Core Laboratory Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, Sichuan, China

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Abstract: Chronic myeloid leukemia (CML) can be contextualized as a disease of unregulated self-renewal of stem cells which exist in a quiescent state and are instructed to differentiate and mobilize to circulation under pathologic circumstances leading to tumor invasion and metastasis. Here we found that matrix metalloproteinase-9 (MMP-9), induced by TGF- β 1, upregulated s-KitL and s-ICAM-1, permitting the transfer of c-kit⁺ hematopoietic stem cells (HSCs) from the quiescent to proliferative niche in CML. Further study showed that this MMP-9 production was raised by CML specific BCR/ABL⁺ oncogene mediated TGF- β 1. Besides, phosphatidylinositol-3 kinase (PI3K)/Akt/nuclear factor (NF)- κ B signaling pathway was evidenced to govern this stem cell recruitment in CML pathogenesis. Overall, our observations defined a novel critical role for TGF- β 1 induced PI3K/Akt/NF- κ B signaling pathway in the recruitment of the malignant cells in CML by releasing s-KitL and s-ICAM-1 and this was through a distinct PI3K/Akt/NF- κ B signaling pathway.

Keywords: Chronic myeloid leukemia (CML), mesenchymal stem cell (MSC), hematopoietic stem cell (HSC), matrix metalloproteinase-9 (MMP-9), TGF- β 1, s-KitL, s-ICAM-1

Introduction

Compelling research suggests that a population of cancer stem cells (CSCs) is able to regenerate or self-renew resulting in therapeutic resistance and disease progression; this is particularly true in chronic myeloid leukemia (CML) [1-3]. Local secretion of proteases has been implicated in this tumor-stroma crosstalk. Matrix metalloproteinase-9 (MMP-9) is one of them which has the preferential ability to degrade denatured collagens (gelatin) and collagen type IV, the 2 main components of basement membranes and therefore plays a critical role in tumor progression and metastasis [4]. Previous studies have demonstrated localization of MMP-9 on the plasma membrane of various tumor cells [5-7], recently, the role of MMP-9 in CML pathogenesis has become a focus of attention [8-11]. But the research is mainly focusing on the MMP-9 inducing molecules [12-14] or the effect of the treatment of MMP-9 inhibitors [15]. However, it has become

clear that the role of MMP-9 in CML is much more complex than one of the simple extracellular matrix (ECM) degradation [16]. In clinical trials, many direct MMP-9 inhibitors have not been successfully in treating cancers, therapeutic inhibition of MMP-9 may be achieved by other indirect means such as targeting the signal transduction pathways that regulate MMP-9 expression [17]. Moreover, the regulation of MMP-9 is found to be involved in multiple pathways induced by different kinds of cytokines according to different cell types and illness [18, 19]. Therefore, it is necessary to verify a specific MMP-9 induced pathway in a given cell type.

Therefore, in the present investigation, we aimed to determine the mechanism underlying malignant HSCs recruitment and mobilization. We found that under steady state conditions quiescent c-Kit⁺ HSCs reside in a niche in close contact with stromal cells. Membrane-bound cytokines, such as m-KitL and m-ICAM-1 not

only convey survival signals, but support the adhesion of stem cells to the stroma. CML specific oncogene induced TGF- β 1 activated PI3K/Akt/NF- κ B/MMP9 signaling pathway resulting in the release of s-KitL and s-ICAM-1, enhanced recruitment and mobilization of tumor stem cells to the peripheral circulation.

Material and methods

Reagents

The following antibodies and reagents were used: antiphospho-Akt (Ser473; Calbiochem, San Diego, CA); anti-MMP-9 (Chemicon, Temecula, CA), anti-Akt1/2, mouse immunoglobulin G (IgG) agarose beads, anti-IKK α agarose beads, anti-IKK α , anti-IKK β , and protein A/G agarose beads (Santa Cruz Biotechnology, CA); wortmannin and MG-132; SH-5 (Alexis Biochemicals, San Diego, CA); phosphoglycogen synthase kinase (GSK)-3 α / β (Ser21/9) antibody, GSK-3 fusion protein, kinase buffer, and adenosine 5'-triphosphate (ATP; Cell Signaling Technology, Beverly, MA); elution buffer (Pierce Biotechnology, Rockford, IL); and nuclear extract kit, TransAMTM NF- κ B transcription factor assay kit (Active Motif, Carlsbad, CA).

Patients samples

20 patients with newly diagnosed CML (12 male and 8 female, aged 17-63 years) were recruited in this study. All were Ph⁺ patients with CML in chronic phase as revealed by bone marrow histology and cytogenetic analysis and the immunophenotype of thawed cells was quite variable. None were treated with hydroxyurea or interferon before. The control samples were healthy donors with the equal 20 samples (12 male and 8 female, aged 21-60 years). Their immunophenotype was also quite variable and they were all Ph⁺ negative in BM at donation. All the bone marrow samples were collected after obtaining informed consent according to procedures approved by the Ethics Committee at the 309 Hospital of Peoples Liberation Army.

Cell preparations and culture conditions

Isolation and culture of bone marrow-derived BCR/ABL⁺ MSCs from CML patients were performed as described previously with some modifications [19-21]. Briefly, mononuclear

cells were separated by a Ficoll-Paque gradient centrifugation (specific gravity 1.077 g/mL; Nycomed Pharma AS, Oslo, Norway) and the sorted cells were plated at concentrations of 1 cell/well by limiting dilution in a total of 96 \times 10 wells coated with fibronectin (Sigma, St Louis, MO) and collagen (Sigma) for each patient. Culture medium was Dulbecco modified Eagle medium and Ham F12 medium (DF12) containing 40% MCDB-201 medium complete with trace elements (MCDB) (Sigma), 2% fetal calf serum (FCS; Gibco Life Technologies, Paisley, United Kingdom), 1 \times insulin transferrin selenium (Gibco Life Technologies), 10⁻⁹ M dexamethasone (Sigma), 10⁻⁴ M ascorbic acid 2-phosphate (Sigma), 20 ng/mL interleukin-6 (Sigma), 10 ng/mL epidermal growth factor (Sigma), 10 ng/mL platelet-derived growth factor BB (Sigma), 50 ng/mL fetal liver tyrosine kinase 3 (Flt-3) ligand (Sigma), 30 ng/mL bone morphogenetic protein-4 (Sigma), and 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco Life Technologies) at 37°C and a 5% CO₂ humidified atmosphere. Culture media were changed every 4 to 6 days.

RT-PCR

RNA isolation and reverse transcription were performed as previously described [32]. Oligonucleotide primer sequences were as follows: β -actin (264 bp), forward: 5'-GAGACCTCAACACCCCAGCC-3'; reverse: 5'-AATGTCACGCACGATTTCCC-3'; s-KitL (263 bp), forward: 5'-AGAGGTCTCAGAAGGGACCG-3', reverse: 5'-GGGCCATACAGGACACGAAG-3'; s-ICAM-1 (167 bp), forward: 5'-TGCTTTTCCAGGGGTGTGTT-3', reverse: 5'-TACTTCTGCACTAATTTGGCA-3'; TGF β 1 (198 bp), forward: 5'-GGAAACCCACAACGAAATCTATGAC-3', reverse: 5'-TTGCTGAGGTATCGCCAGGAAT-3'; TGF β 2 (213 bp), forward: 5'-CGCCAAGGAGGTTTACAAAATAGAC-3', reverse: 5'-TCAATCCGTTGTTTCAGGCACTCT-3'; TGF β 3 (233 bp), forward: 5'-CTGGCGGAGCACAACGAACT-3', reverse: 5'-AGGATATCTCCATTGGGCTGAAAG-3'; MMP-9 (201 bp), forward: 5'-TCCC-CATCGCCATCCCC-3' reverse: 5'-CACCATGGCC-TCGGCTGG-3'. To all the above genes, amplification was performed under the same cycling conditions (1 minute at 94°C, 50 seconds at 57°C, 1 minute at 72°C), except the number of cycles that were specified for each gene (31 for s-KitL, s-ICAM-1, TGF β 1, TGF β 2 and TGF β 3, 32 for MMP-9).

Hematopoietic stem cells in CML

Co-culture assay

Fik1⁺CD31⁻CD34⁻ MSCs were isolated from healthy donors and CML patients were cultured in T25 bottle respectively and regulated the number to 1×10⁶/bottle. They were radiated 10 Gy/unit 1.5 hours later and then mononuclear cells from cord blood were collected and cultured with the MSCs in T25 bottle to form the co-culture system. Collected 2.5 ml medium and filled 2.5 ml H5100 into it a week later. Centrifuged the HSCs and collected supernatant, using the 1640 to regulate the cell number into 1×10⁶/well. Put CFU-mix 300 μl/well into 24 well plate and collected the supernatant 100 μl/well for further assay. After a 14-day incubation at 37°C in 5% CO₂, CFU-GMs were scored as aggregated of more than 50 cells.

Western blot and immunoprecipitation

MSCs were harvested at specific times after treatment with regents as indicated in each experiment and then were mixed with loading buffer and separated, after electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Pall Filtron) using a semidry blotting apparatus (Pharmacia) and probed with mouse monoclonal antibodies to indicated amount, followed by incubation with peroxidase-labeled secondary antibodies. Detection was performed by the use of a chemiluminescence system (Amersham) according to the manufacturer's instructions. Then membrane was stripped with elution buffer and reprobed with antibodies against the nonphosphorylated protein as a measure of equal loading. Controls for the immunoprecipitation used the same procedure, except agarose beads contained only mouse IgG.

T lymphocyte activity assay

Cells isolation and culture were performed as above mentioned and we divided them into 3 groups: TS: PHA induced T lymphocyte; TS+ healthy MSCs: the healthy MSCs and PHA induced T lymphocyte; TS+Fik1⁺ MSC: Fik1⁺ MSC and PHA induced T lymphocyte. The proportion of MSCs and T lymphocytes was 1:10; CD69, CD25 and CD44 were examined 12, 24 and 72 hrs later respectively.

Fluorescence activated cell sorting (FACS)

For immunophenotype analysis, expanded clonal cells were stained with antibodies

against Fik1, CD29, CD31, CD34, CD44, CD45, CD105, HLA-ABC, vWF (all from Becton Dickinson Immunocytometry Systems, Mountain View, CA). For intracellular antigen detection, cells were first fixed in 2% paraformaldehyde (Sigma) for 15 minutes at 4°C and permeabilized with 0.1% saponin (Sigma) for 1 hour at room temperature. Cells were washed and labeled with fluorescein isothiocyanate (FITC) conjugated secondary goat antimouse, goat antirabbit, or sheep antigoat antibodies (Sigma), then washed and analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA).

FISH analysis

We cultured BCR/ABL⁺ MSCs from male CML patients (n = 12) and Y chromosome was detected using a probe (CEP Y Spectrum Red; Vysis, Downers Grove, IL) according to the manufacturer's instructions. Normal cells showed 2 red able signals and 2 green bar signals. BCR/ABL⁺ MSCs showed a single red and a single green signal representing normal able and bar genes and the yellow signal representing fusion of able and bar genes.

RNA-i experiments

The is-RNA sequence targeting human MMP-9 chosen in this study (from mRNA sequence; Nitrogen online) corresponds to the coding region 377-403 relative to the first nucleotide of the start codon (target = 5'-AAC ATC ACC TAT TGG ATC CAA ACT AC-3'). Computer analysis using the software developed by Ambion Inc. confirmed this sequence to be a good target. si-RNAs were 21 nucleotides long with symmetric 2-nucleotide 3'overhangs composed of 2'-deoxythymidine to enhance nuclease resistance. The si-RNAs were synthesized chemically and high pressure liquid chromatography purified (Genset, Paris, France). Sense si-RNA sequence was 5'-CAUCACCUAUUGGAUCCAA-dTdT-3'. Antisense si-RNA was 5'-UUGGAUCCAAUAGGUGAUGdTdT-3'. For annealing of si-RNAs, mixture of complementary single stranded RNAs (at equimolar concentration) was incubated in annealing buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, and 10 mM MgCl₂) for 2 minutes at 95°C followed by a slow cooling to room temperature (at least 25°C) and then proceeded to storage temperature of 4°C. Before transfection, cells cultured at 50% confluence in

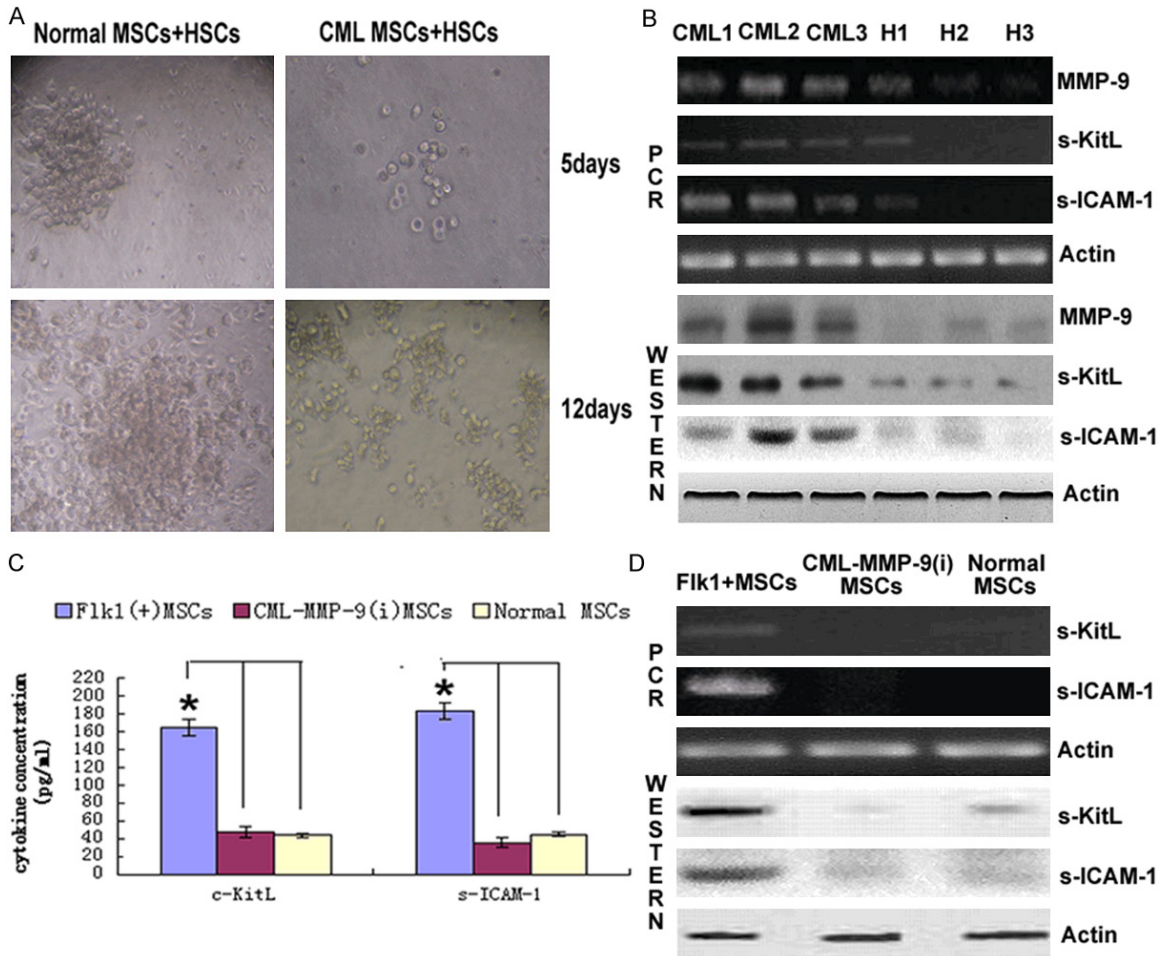


Figure 1. MMP-9 mediated release of KitL enhanced HSCs proliferation (A) Co-culture of BCR/ABL⁺ MSCs and healthy MSCs with HSCs respectively showed the number of GM-CFC (colony forming unit-common precursor of granulocyte and monocyte) in BCR/ABL⁺ MSCs with HSCs group formed more clusters and aggregates than the control one. (B) Supernatants from the co-culture were also collected and MMP-9 was analyzed by ELISA. Columns represent cytokine concentration mean \pm SD of 3 different experiments, * $P < 0.05$. (C) MSCs from BCR/ABL⁺ CML and healthy donors expressed different levels of MMP-9 and KitL assessed by Western blot and RT-PCR. The results were representative of at least 3 independent experiments. (D) Western blot and RT-PCR assayed the level of KitL of CML MSCs, CML-MMP-9 (i) MSCs and healthy donor MSCs respectively. Columns represent cytokine concentration mean \pm SD of 3 different experiments, * $P < 0.05$.

6-well plates (10 cm²) were washed two times with OPTIMEM 1 (Invitrogen) without FCS and incubated in 1.5 ml of this medium without FCS for 1 hour. Then, cells were transfected with MMP-9-RNA duplex formulated into Mirus TransIT-TKO transfection reagent (Mirus Corp, Interchim, France) according to the manufacturer's instructions. Unless otherwise described, transfection used 20 nM RNA duplex in 0.5 ml of transfection medium OPTIMEM 1 without FCS per 5 \times 10⁵ cells for 6 hours and then the medium volume was adjusted to 1.5 ml per well with RPMI 2% FCS. SilencerTM neg-

ative control 1 si-RNA (Ambion Inc.) was used as negative control under similar conditions (20 nM). The efficiency of silencing is 80% in our assay.

Enzyme-linked immunoadsorbent assays

This was carried out according to the manufacturer's (Oncogene Research Products) recommendations. Results were compared with those obtained with serially diluted solutions of commercially purified controls. Briefly, anti-human cytokine antibodies (R & D Systems, Minneapolis, MN) was added at 0.4 μ g/ml in 0.05 M

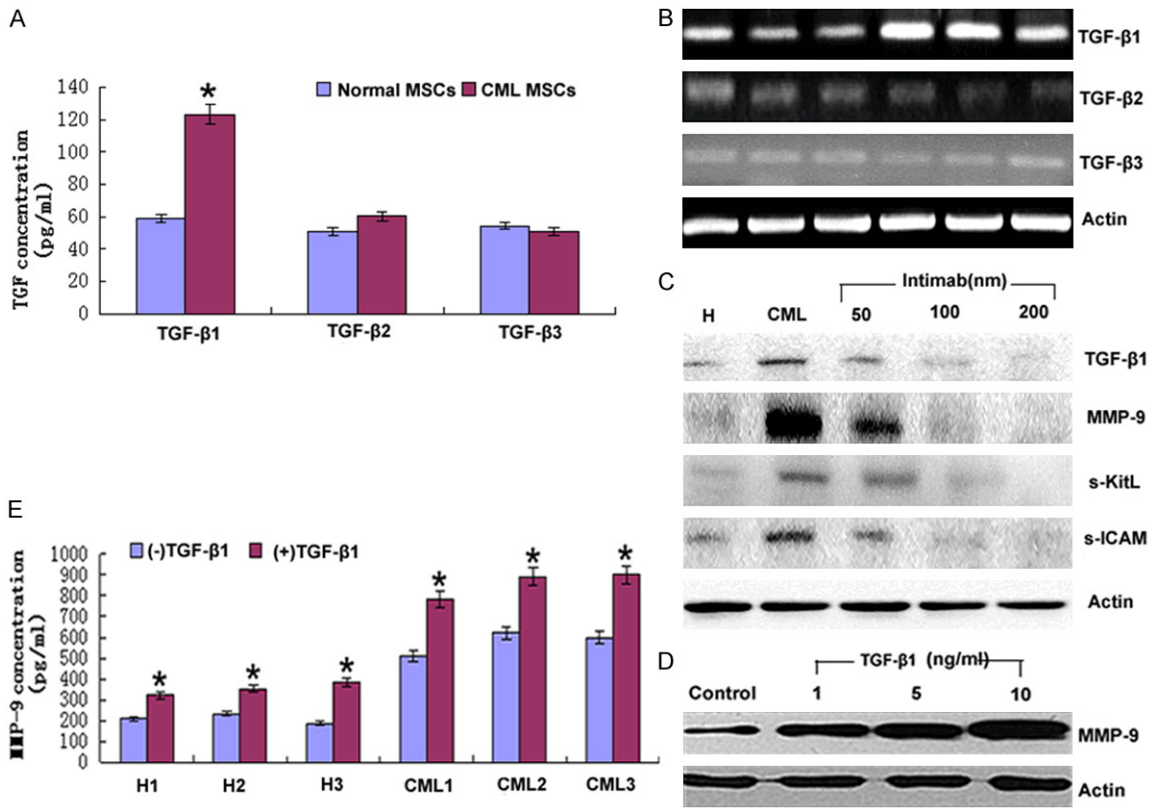


Figure 2. BCR/ABL tyrosine kinase induced TGF-β1 upregulated MMP-9 in CML. (A) The supernatants from BCR/ABL⁺ MSC and healthy donors MSCs were collected to do ELISA to examine the expression of the 3 isotype of TGF-β and the results were representative of at least 3 independent experiments. *P<0.05. (B) RT-PCR analyzed the mRNA level of 3 isotype of TGF-β in BCR/ABL⁺ MSCs and healthy donors. The results were representative of at least 3 independent experiments. (C) BCR/ABL⁺ MSCs were treated with the indicated amount of Intimab for 2 hours, an inhibitor of BCR/ABL oncogene and then cell lysates were analyzed by Western blot with GAPDH as a measure of equal sample-loading amount. (D) BCR/ABL⁺ MSCs were treated with the indicated amount of TGF-β1 and then cell lysates were analyzed by Western blot with GAPDH as a measure of equal sample-loading amount and (E) Supernatants were also collected and MMP-9 was analyzed by ELISA. Columns represent cytokine concentration mean ± SD of 3 different experiments, * P<0.05.

bicarbonate buffer (pH 9.3) to 96-well, U-bottom, polyvinyl microplates (Becton Dickinson and Co., Oxnard, CA) and the cell number was 1×10⁵/100 μl. After incubation overnight at 4°C, the plates were washed and blocked with 1% gelatin for 1 hour. Samples (50 μl) or standard protein diluted in 0.5% gelatin were added to the wells. After incubation for 1 hour at 37°C, the plates were washed again, and 50 ng/ml biotinylated antimouse antibody (R & D Systems) was added for 1 hour at 37°C. The plates were then washed and incubated with streptavidin-HRP for 1 hour at 37°C. After washing, 0.2 mM ABTS (Sigma Chemical Co.) was added to the wells, and after 10 minutes, the colorimetric reaction was measured at 405 nm with an ELISA reader VERSAmax (Molecular Devices, Sunnyvale, CA).

Preparation of nuclear extracts and the electrophoretic mobility shift assay (EMSA)

BCR/ABL⁺ MSCs or cells pre-treated as indicated were harvested, and then nuclear extracts were prepared as described previously. Oligonucleotides corresponding to the downstream NF-κB binding sequences 5'-AGT TGA GGG GAC TTT CCC AGG C-3' were synthesized, annealed and end-labeled with [³²P] ATP using T4 poly-nucleotide kinase, and EMSA was performed as described previously [22].

Akt kinase assay

Akt kinase assay was performed as described previously with some modifications [22]. MSC were harvested at specific times after treat-

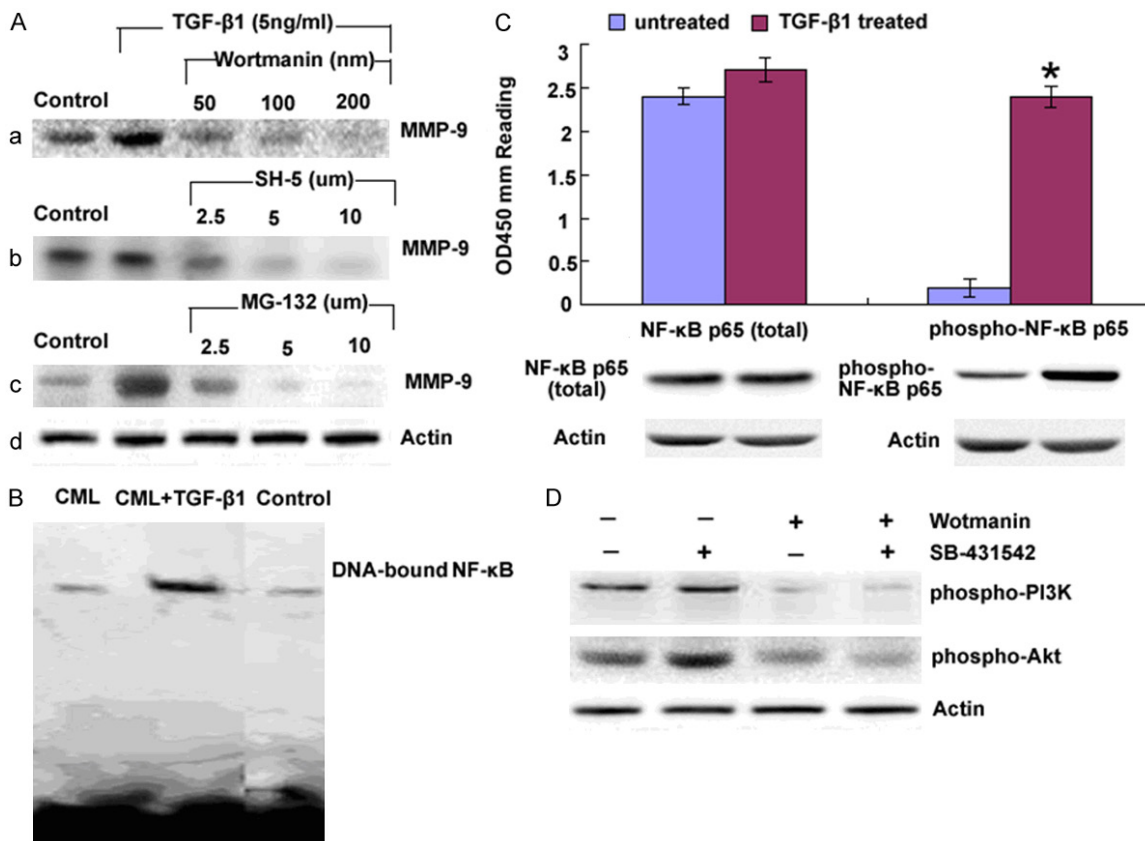


Figure 3. TGF-β1 upregulated MMP-9 production via PI3K/Akt/NF-κB. A. BCR/ABL⁺ MSCs were treated with (a) Wortmanin (b) SH-5 and (c) MG-132 for 1 hour prior to the addition of TGF-β1 as the indicated amounts. Culture supernatants were harvested after 48 hours and analyzed for MMP-9 by Western blot, Me2SO was also added to one of the cultures as a control. B. Cell nuclear extracts were prepared and the DNA binding ability of NF-κBp65 was examined by EMSA. C. Nuclear and cytoplasmic protein were collected respectively to assay the total and phosphorylated NF-κBp65 by Western blot and ELISA (PathScan phospho-NF-κBp65 Sandwich ELISA kit) in the presence or absence of TGF-β1 treatment. OD450 reading was shown in the top figure and the corresponded Western blot using phospho-NF-κBp65 (Ser536) rabbit mAb (right panel) or NF-κBp65 antibody (left panel) was shown in the bottom figure. Columns represent cytokine concentration mean ± SD of 3 different experiments, *P<0.05. D. Protein from BCR/ABL⁺ MSC treated with Wortmanin, an inhibitor of PI3K and SB-431542, an inhibitor of Smad respectively and phosphorylated PI3K and Akt were examined by Western blot.

ment with reagents and lysed in lysis buffer. After equalizing the protein to 1.0 mg/200 μl in a 1.5-ml Eppendorf tube, 10 μl Akt antibody was added, and the samples were rotated overnight at 4°C. Then 20 μl protein A/G agarose bead slurry was added, and the samples were rotated at room temperature for 2 hours. The beads were washed twice with lysis buffer and kinase buffer, and the samples were suspended in 50 μl kinase buffer supplemented with 1 μl 10 mM ATP and 1 μg GSK-3 fusion protein and incubated at 30°C for 30 minutes. The phosphorylation of GSK-3 fusion protein was analyzed by Western blot with phosphor-GSK-3α/β (Ser2119) antibodies.

NF-κB p65 activation assay

MSC were harvested at specific times after treatment with reagents, and the nuclear extracts and the activation assay of p65 were performed according to the instructions of the manufacturer of the TransAM™/NF-κB kit. Briefly, 2 μg/well nuclear extract protein was added to a microtiter plate coated with a specific oligonucleotide of p65. The coated plate was then incubated for 1 hour at room temperature with mild agitation, after which a primary antibody recognizing the p65 was added, and the plate was incubated for an additional 1 hour at room temperature. Anti-IgG horseradish peroxidase-conjugated secondary antibody

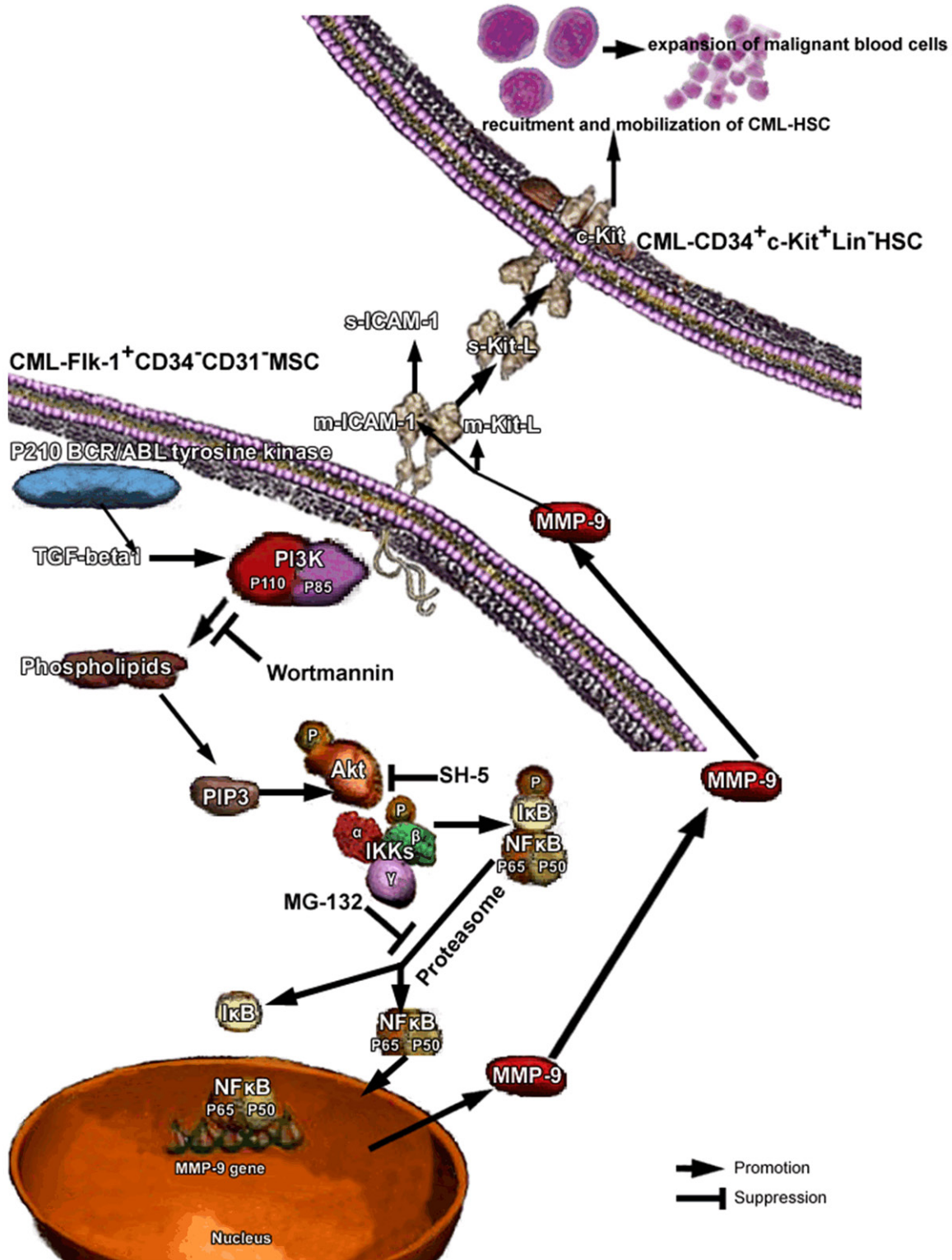


Figure 4. Recruitment of HSCs in CML niche requires TGF-beta1 activated PI3K/Akt/NF-κB/MMP9 signaling pathway. Under steady state conditions quiescent c-Kit⁺ HSCs reside in a niche in close contact with stromal cells. Membrane-bound cytokines, such as mKitL and mICAM-1 not only convey survival signals, but support the adhesion of stem cells to the stroma. CML specific oncogene induced TGF-beta1 activated PI3K/Akt/NF-κB/MMP9 signaling pathway resulting in the release of s-KitL and s-ICAM-1, enhanced recruitment and mobilization of tumor stem cells to the peripheral circulation.

was then added, and the plate was incubated for 1 hour at room temperature. At the end of the hour, the developing and stop solution were added, and an optical density of 450 nm (OD450) was read on a Wallac Victor 1420 multilabel counter (Perkin Elmer Life Sciences, Shelton, CT). Three duplicates were done for each sample.

Statistical analysis

Results are expressed as mean \pm standard deviation. Data were analyzed using the unpaired two-tailed student's t test and the log rank test. *P* values of <0.05 were considered significant.

Results

BCR/ABL⁺ MSCs promoted recruitment of HSCs through s-KitL and s-ICAM-1 upregulated by MMP-9

Co-culture of BCR/ABL⁺ MSCs and normal MSCs with HSCs showed that the number of GM-CFU (colony forming unit-common precursor of granulocyte and monocyte) in BCR/ABL⁺ MSCs with HSCs group was lesser than those in healthy donors with HSCs group and lost the support at the 3rd week comparing with the control group, and in the longer period of co-culture, BCR/ABL⁺ MSCs group showed more clusters and less aggregates (**Figure 1A**). Further studies showed that MMP-9, s-KitL and s-ICAM-1 in BCR/ABL⁺ MSCs were higher than those in healthy donor MSCs evidenced by Western and PCR (**Figure 1B**). The results indicated that BCR/ABL⁺ MSCs secreted abnormally higher MMP-9, s-KitL and s-ICAM-1 comparing with the control group. To investigate whether the s-KitL and s-ICAM-1 were MMP-9 dependent, we used transfection of a double stranded RNA that targeted the MMP-9 mRNA into bMSC to deplete the corresponding mRNA and protein and this extinction did result in down-regulation of s-KitL and s-ICAM-1 illustrated by Western blot, PCR and ELISA (**Figure 1C, 1D**).

Abl kinase upregulated TGF- β 1 in CML

To investigate the mechanism involved in MMP-9 synthesis, we first assayed the expression of TGF- β in CML and healthy donors from mRNA and protein level respectively. As TGF- β has 3 isotype, we examined the expression of

them to ensure if there existed any differences and the results indicated that TGF- β 1 was apparently higher than TGF- β 2 and TGF- β 3 as shown by ELISA, PCR and Western blot (**Figure 2A, 2B**) which inspired us to do further research on TGF- β 1. We then examined the relationship between CML specific BCR/ABL oncogene and TGF- β 1. Intimab, inhibitor of BCR/ABL oncogene, blocked the induction of TGF- β 1 in a dose dependent manner (**Figure 2C**). Furthermore, the effect of various doses of TGF β 1 on MMP-9 production by CML MSCs was assessed to correlate the MMP-9 levels with the induction of signaling components leading to the synthesis of the enzyme (**Figure 2D**). Results from ELISA (**Figure 2E**) showed TGF- β 1 upregulated MMP-9 production both in CML and healthy donors. Those results demonstrated that BCR/ABL oncogene and TGF- β 1 were involved in the regulation of MMP-9 production.

TGF- β 1 upregulated MMP-9 production via PI3K/Akt/NF- κ B

To determine the involvement of PI3K signaling pathway in BCR/ABL⁺ MSCs, we initially examined the effect of PI3K activity on the induction of MMP-9 by TGF- β 1. The addition of Wortmanin, inhibitor of PI3K, SH-5, inhibitor of Akt and MG-132, inhibitor of NF- κ B, suppressed the MMP-9 synthesis induced by TGF- β 1 respectively (**Figure 3A**). We then analyzed nuclear extraction by EMSA for DNA binding activity of NF- κ B. As shown by **Figure 3B**, lane 1, 2 and 3 represented the results of nuclear extraction of BCR/ABL⁺ MSCs, BCR/ABL⁺ MSCs with 5 ng/ml TGF- β 1 pre-culturing for 2 hours and healthy donors respectively and the results indicated that NF- κ B did involved in TGF- β 1 induced MMP-9 production. We at the same time analyzed total cytoplasmic NF- κ Bp65 and phosphorylated NF- κ Bp65 in the presence or absence of TGF- β 1 (5 ng/ml) treatment using Western blot and ELISA (**Figure 3C**) and the results showed that TGF- β 1 upregulated phosphorylated NF- κ Bp65 while did little effects on total NF- κ Bp65 production.

Discussion

Chronic myeloid leukemia is a clonal hematopoietic stem cell disorder characterized by the t (9;22) chromosome translocation and resultant production of the constitutively activated BCR/ABL tyrosine kinase [23]. Although Interferon- α ,

Intimab(a *BCR/ABL* tyrosine kinase inhibitor) and stem cell transplantations are the standard therapeutic options, transplant-related morbidity from graft-versus-host disease and mortality rates of 10% to 20% have greatly reduced the allogeneic hematopoietic cell transplantation in clinics [24], while interferon- α is only effective in some patients to some degree and chemotherapeutic intervention does not result in prolonged overall survival [25, 26] and the reason is possibly due to some unknown biology of the CML cancer stem cells [27].

Our laboratory has identified the Flk-1⁺CD34⁺CD31⁻ MSCs in CML as the cancer stem cells, based on this concept, we first examined the biological characteristics of them. FISH analysis of the Flk-1⁺CD34⁺CD31⁻ MSCs derived from CML bone marrow showed that they were BCR/ABL⁺ which indicated that this oncogene had already mutated at the very stem cell level. More importantly, as normal MSCs have the ability to inhibit T lymphocytes at the stage of G₀/G₁, while our research showed that the ability of CML-derived MSCs on T lymphocytes activity inhibition became attenuated. This indicated on one hand, the immune regulatory ability of CML MSCs became decreased, on the other hand, as the basis of immunological cells to destroy the tumor cells was to recognize the cell surface molecules and the abnormalities of these molecules would lead to false recognition and cleanse of malignant cells which was called immune evasion, so, in CML, there must exist molecules deformities. Meanwhile, co-culture assay indicated CML-derived MSCs existed adhesion deficiency, as HSCs, and other stem cells including cardiac (Orlic et al., 2001), endothelial (Peichev et al., 2000), and epithelial cells express c-Kit, the receptor for KitL, suggesting a common signaling cascade may govern their proliferation and recruitment. Besides, a lot of blood disease such as ALL, CLL and MDS are found to express high level of s-ICAM-1 and clinical researches indicated the level of it correlated directly with disease stage, prognosis and survival period [28-30]. All these inspired us to further examine the s-KitL and s-ICAM-1, and both of them were higher in CML than normal donor. So, we believed that enhanced expression of s-ICAM-1 facilitated on the one hand, tumor cells immune evasion by blocking the ICAM/LFA connection and made them fail to be recognized by T lymphocyte and

NK cells; on the other hand, degraded the adhesion of c-kit⁺ HSCs with bone marrow matrix and destroy the protective screen between hematopoietic region (endothelial cells and matrix) and peripheral circulation, while at the same time, increased s-KitL promoted c-kit⁺ HSCs in CML recruitment and mobilization, resulting in extramedullary leukemia cells infiltration. Besides, it further confirmed our belief that purified true leukemia stem cells could provide a target for immune-based therapies and biologic response modifiers [31].

MMP-9 is important for maintaining normal hematopoietic process, abnormally higher MMP-9 will degrade extra-cellular matrix and change the cytokines and molecules that have bearing with HSCs adhesion and motivation. In recent years, MMP-9 is found to do effect on cells recruitment and differentiation [32-34]. In the present investigation, we evidenced that MMP-9 had already expressed at the cancer stem cell level which was the first time as other documents concerned mainly about the leukemia cells. We also found that the s-KitL and s-ICAM-1 was MMP-9 dependent proved by RNA interference in Flk1⁺ CML MSCs.

So, to further investigate the mechanism behind MMP-9 production in BCR/ABL⁺ CML, we examined the expression of the 3 isotypes of TGF β in CML, TGF- β 1 was abnormally higher in BCR/ABL⁺ CML comparing with that in healthy donors. Thereby, in the next research, we focused on the relationship between BCR/ABL oncogene and TGF- β 1. We found that Intimab, inhibitor of BCR/ABL oncogene, blocked the induction of TGF- β 1 in a dose dependent manner. Further evidence shows that TGF- β 1 upregulated MMP-9 also in a dose dependent manner. All these data indicated that BCR/ABL oncogene induced TGF- β 1 which upregulated MMP-9 production in CML. While, one intricate aspect about TGF- β 1 is that although Smad is regarded as the classic regulating pathway, more and more evidence shows that some distinct responses are also involved in its regulation which can be classified as Smad independent pathway, such as Ras [35], Rho proteins [36], extracellular signaling kinase [37] and p38 [38]. Recently, phosphatidylinositol 3-kinase (PI3K) is found to be involved in TGF- β 1 signaling positively or negatively at least in some cell types as both stimulation and inhibition effects have been described [39-41]. To

investigate whether TGF- β 1 was Smad dependent or independent in BCR/ABL⁺ MSCs, we used Smad and PI3K inhibitor respectively as indicated and the results showed that it was directed by PI3K instead of Smad in CML. Actually, TGF- β and PI3K are involved in many cell lines [42-45].

It was reported that PI3K was activated by tyrosine kinase [46-48] and our results further verified that in CML, it was activated by tyrosine kinase induced TGF- β 1. Following its recruitment to these receptors in the plasma membrane, PI3K is activated and phosphorylates to generate the second messenger PIP3, whose levels are tightly regulated by the action of phosphatases. PIP3 does not activate Akt directly but instead, appears to recruit Akt to the plasma membrane and to alter its conformation to allow subsequent phosphorylation by the phosphoinositide-dependent kinase [49, 50]. When the capability for PI3K to produce PIP3 was blocked in BCR/ABL⁺ MSCs by wortmannin, MMP-9 production was inhibited which indicated that the PI3K pathway had a central role in regulation of CML MMP-9 synthesis.

Furthermore, when SH-5 blocked Akt phosphorylation specifically, MMP-9 production was also inhibited. These data showed that PIP3 mediated MMP-9 production in BCR/ABL⁺ MSC, primarily through Akt and not other downstream components of PI3K containing a PH domain. MG-132 inhibition of MMP-9 production indicated that NF κ B was also involved in this pathway which coordinated with the reports of other cell lines [51].

Three members of the IKK family have been isolated, and these are now referred to as IKK1/IKK α , IKK2/IKK β and IKK3/IKK γ [52]. Expression and the ratio of IKK α to IKK β vary among cell types. NF κ B, in the cells with a high proportion of IKK α to IKK β , is sensitive to Akt activity [53]. Our data showed that IKK α was predominant in human BCR/ABL⁺ MSCs, which was apparently different from that in healthy donors where the 3 subunits presented no big differences. Based on this data, we hypothesized that Akt might bind to IKK α constitutively and their phosphorylation might occur following with TGF- β 1 stimulation. Immunoprecipitation confirmed our idea which showed that the phosphorylation of Akt was regulated by PI3K and Akt subsequently phosphorylated IKK α which regulated the activation of NF- κ B.

Finally, the I κ B proteins are degraded rapidly by the proteasome complex once ubiquitinated, thereby freeing NF- κ B, which then enters the nucleus, binds to DNA, and activates transcription [54]. It had been shown that Akt regulated NF- κ B activation directly through the activation of IKK α and our findings from 2 aspects, EMSA and Western blot, demonstrated that the PI3K/Akt pathway also regulated I κ B degradation and activation of NF- κ B in BCR/ABL⁺ MSC stimulated by TGF- β 1.

Generally speaking, as what is schematically represented in **Figure 4**, we demonstrated a decisive checkpoint for MMP-9 to rapidly release the stem cell-active cytokines s-KitL and s-ICAM-1, thereby directing tumor cell immune evasion and mobilization to extramedullary infiltration. What more, the mechanism underlying this niche switch depended on the upregulation of CML specific BCR/ABL oncogene mediated PI3K/Akt/IKK α /NF- κ B signaling pathway. A intriguing aspect of this study is the illustration of stem cell-stromal cell interactions being amplified by membrane-anchored cytokines which are mediated by a specific oncogene induced signaling pathway. Modulation of local cytokines change the HSCs fates provide promising hope for clinical targeting therapy.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiaodong Wang, Department of Core Laboratory Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital, 32# W. Sec 2, 1st Ring Rd. Chengdu, Sichuan, China. E-mail: pumc19780@163.com

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