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TGF-β1-induced PI3K/Akt/NF-κB/MMP9 signalling pathway is activated in Philadelphia chromosome-positive chronic myeloid leukaemia hemangioblasts

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Overwhelming evidence from chronic myeloid leukaemia (CML) research indicates that patients harbour quiescent CML stem cells that are responsible for blast crisis. While the haematopoietic stem cell (HSC) origin of CML was first suggested over 30 years ago, recently CML-initiating cells beyond HSCs are also being investigated. We have previously isolated fetal liver kinase-1-positive ($Flk1^+$) cells carrying the BCR/ABL fusion gene from the bone marrow of Philadelphia chromosome-positive (Ph^+) patients with hemangioblast property. Here, we show that these cells behave abnormally comparing with the hemangioblasts in healthy donors. These Ph^+ putative CML hemangioblast up-regulated TGF-β1 and result in activating matrix metalloproteinase-9 to enhance s-KitL and s-ICAM-1 secretion. Further studies showed that phosphatidylinositol-3 kinase (PI3K)/Akt/nuclear factor-κB signalling pathway was involved in CML pathogenesis. These findings provide direct evidence for the first time that hemangioblasts beyond HSCs play a critical role in the progression of CML.

Keywords: chronic myeloid leukaemia (CML)/hemangioblasts/Hematopoietic Stem Cell (HSC)/Matrix Metalloproteinase-9 (MMP-9)/transforming growth factor-beta 1(TGF-β1).

Abbreviations: CML, chronic myeloid leukemia; HSC, hematopoietic stem cell; MMP-9, matrix metalloproteinase-9; TGF-β1, transforming growth factor-beta 1.

More and more research shows that a clonal population of cancer stem cells plays important role in the pathology of disease progression: this is particularly true in chronic myeloid leukemia (CML) (*1–3*). The CML-specific Philadelphia chromosome and the subsequent determination of its BCR/ABL fusion oncogene product helps the further investigation of the pathogenesis of CML. Target therapy has been an important method to reduce mortality rates of CML patients, but the disease progression to blast crisis which may be caused by cancer stem cells is still the main death reason. Under steady-state conditions, these cancer stem cells are in an undifferentiated and quiescent state and localized in the stem cell 'niche' which are critical for cells progression and development, yet molecular mechanisms underlying quiescent stem cells in these specialized 'niche' and why and how these cells are differentiated to affect CML progression are still not reported.

Local secretion of proteases has been implicated in this tumour-stroma cross-talk. Matrix metalloproteinase-9 (MMP-9) is one of the proteases that has the preferential ability to degrade denatured collagens (gelatin) and collagen type IV, the two main components of basement membranes and therefore plays a critical role in tumour progression and metastasis (*4*). Previous studies have demonstrated localization of MMP-9 on the plasma membrane of various tumour cells (*5–7*) and 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 MMP-9 inhibitors (*15*). However, it has become clear that the role of MMP-9 in CML is not limited to simple extracellular matrix (ECM) degradation (*16*). The regulation of MMP-9 is found to be involved in multiple pathways induced by different kinds of cytokines in different cell types and illness (*17*, *18*). Therefore, it is necessary to verify a specific MMP-9 induced pathway in a given cell type.

Previous studies (*19*) from our laboratory have identified fetal liver kinase-1 positive ($Flk1^+$) cells carrying the BCR/ABL fusion gene from the bone marrow of Philadelphia chromosome positive (Ph^+) patients with CML and found that these cells could differentiate into malignant blood cells and phenotypically defined endothelial cells at the single-cell level, suggesting these cells have the properties of hemangioblasts. 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 BCR/ABL oncogene up-regulated TGF-β1 expression, then the released TGF-β1 activated PI3K/Akt/NF-κB/MMP9 signalling pathway and subsequently resulted in the release of s-KitL and s-ICAM-1, which ultimately enhanced the recruitment and mobilization of tumor stem cells to the peripheral circulation.

Materials and Methods

Reagents

The following antibodies and reagents were used: anti-phospho-Akt (Ser473; Calbiochem, San Diego, CA, USA); anti-MMP-9 (Chemicon, Temecula, CA, USA); anti-Akt1/2, mouse immunoglobulin G (IgG) agarose beads, anti-IKKα agarose beads, anti-IKKα, anti-IKKβ, protein A/G agarose beads, anti-phoshpo-PI3K (Santa Cruz Biotechnology, CA, USA); HSc025, Wortmannin, SN-50, SH-5, anti-GAPDH, anti-phospho-SMAD2 (Alexis Biochemicals, San Diego, CA, USA); phosphoglycogen synthase kinase (GSK)-3α/β (Ser21/9) antibody (Chemicon); anti-TGF-β1, anti-s-KitL, GSK-3 fusion protein, kinase buffer, anti-s-ICAM-1anti-Actin, anti-NF-kB, anti-phoshpo-NF-kB, SB431542, adenosine 5′-triphosphate (ATP; Cell Signaling Technology, Beverly, MA, USA); elution buffer (Pierce Biotechnology, Rockford, IL, USA); nuclear extract kit, TransAMTM NF-κB transcription factor assay kit (Active Motif, Carlsbad, CA, USA).

Patient samples

Twenty patients with newly diagnosed CML (12 males and 8 females, 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. None was treated with hydroxyurea or interferon before. The control samples were from 20 healthy donors (12 males and 8 females, aged 21–60 years). Bone marrow samples were collected after obtaining informed consent according to procedures approved by the Ethics Committee at the 309th Hospital of Peoples Liberation Army.

Cell preparations and culture

Isolation and culture of bone marrow-derived CML hemangioblasts were performed as described previously (*19–21*).

RT–PCR

RNA isolation and reverse transcription were performed as previously described (*22*). Oligonucleotide primer sequences were as follows: β-actin (264 bp), forward: 5′-GAG ACC TTC AAC ACC CCA GCC-3′; reverse:5′-AAT GTC AC G CAC GATT TCC C-3′; s-KitL (263 bp), forward: 5′-AGA GGT CTC AGA AGG GAC CG-3′, reverse: 5′-GGG CCA TAC AGG ACA CGA AG-3′; s-ICAM-1(167 bp), forward: 5′-TGC TTT TTC CAG GGG TGT GTT-3′, reverse: 5′-TAC TTC CTG CAC TAA TTT GGC A-3′; TGF-β1 (198 bp), forward: 5′-GGA AAC CCA CAA CGA AAT CTA TGA C-3′, reverse: 5′-TTG CTG AGG TAT CGC CAG GAA T-3′; TGF-β2 (213 bp), forward: 5′-CGC CAA GGA GGT TTA CAA AAT AGA C-3′, reverse: 5′-TCA ATC CGT TGT TCA GGC ACT CT-3′; TGF-β3 (233 bp), forward: 5′-CTG GCG GAG CAC AAC GAA CT-3′, reverse: 5′-AGG ATA TCT CCA TTG GGC TGA AAG-3′; MMP-9 (201 bp), forward: 5′-TCC CCA TCG CCA TCC CC-3′ reverse: 5′-CAC CAT GGC CTC GGC TGG-3′. For all the above genes, amplification was performed under the same cycling conditions (1 min at 94°C, 50 s at 57°C, 1 min at 72°C), except the number of cycles that were specified for each gene (31 for s-KitL, s-ICAM-1, TGF-β1, TGF-β2 and TGF-β3, 32 for MMP-9).

Western blot and immunoprecipitation

CML hemangioblasts were harvested at specific times after treatment with reagents as indicated in each experiment. Cells were mixed with loading buffer and subject to electrophoresis. After electrophoresis, proteins were transferred to polyvinyl difluoride membranes (Pall Filtron) using a semidry blotting apparatus (Pharmacia) and probed with mouse mAbs, followed by incubation with peroxidase-labelled secondary antibodies. Detection was performed by the use of a chemiluminescence system (Amersham) according to the manufacturer's instructions. Then membrane was striped with elution buffer and reprobed with antibodies against the non-phosphorylated protein as a measure of loading control. Controls for the immnoprecipitation used the same procedure, except agarose beads contained only mouse IgG.

T lymphocyte activity assay

Cells isolation and culture were performed as above mentioned. Harvested cells were stimulated with PHA at the presence or absence of CML hemangioblasts. The ratio of hemangioblasts to T lymphocytes was 1:10. CD69, CD25 and CD44 were examined 12, 24 and 72 h later, respectively.

FISH analysis

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

Fluorescence-activated cell sorting

For immunophenotype analysis, expanded clonal cells were stained with antibodies against Flk1, CD29, CD31, CD34, CD44, CD45, CD105 (all from Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). For intracellular antigen detection, cells were first fixed in 2% paraformaldehyde (Sigma) for 15 min at 4°C and permeabilized with 0.1% saponin (Sigma) for 1 h at room temperature. Cells were washed and labelled with fluorescein isothiocyanate (FITC) conjugated secondary goat anti-mouse, goat anti-rabbit or sheep anti-goat antibodies (Sigma), then washed and analysed using a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

RNA-i experiments

RNA-i experiments was performed as described previously (*23*).

Enzyme-linked immunoadsorbent assays

This was carried out according to the manufacturer's recommendations (Oncogene Research Products). Results were compared with those obtained with serially diluted solutions of commercially purified controls. Anti-human cytokine antibodies (R&D Systems, Minneapolis, MN, USA) was added at 0.4 μg/ml in 0.05 M bicarbonate buffer (pH 9.3) to 96-well, U-bottom, polyvinyl microplates (Becton Dickinson and Co., Oxnard, CA, USA) and the cell number was 1×10^5/100 μl. After incubation overnight at 4°C, the plates were washed and blocked with 1% gelatin for 1 h. Samples (50 μl) or standard protein diluted in 0.5% gelatin were added to the wells. After incubation for 1 h at 37°C, the plates were washed again, and 50 ng/ml biotinylated anti-mouse antibody (R&D Systems) was added for 1 h at 37°C. The plates were then washed and incubated with streptavidin-HRP for 1 h at 37°C. After washing, 0.2 mM ABTS (Sigma Chemical Co.) was added to the wells, and after 10 min, the colorimetric reaction was measured at 405 nm with an ELISA reader VERSAmax (Molecular Devices, Sunnyvale, CA, USA).

Preparation of nuclear extracts and the electrophoretic mobility shift assay

CML hemangioblasts or cells pre-treated as indicted were harvested, then nuclear extracts were prepared. Oligonucleotides corresponding to the downstream NF-kB binding sequences 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ were synthesized, annealed and end-labelled with [g-32 P] ATP using T4 poly-nucleotide kinase, and electrophoretic mobility shift assay (EMSA) was performed as described previously (*24*).

NF-κB p65 activation assay

Hemangioblasts were harvested at specific times after treatment with reagents. NF-κB p65 activation assay were performed as described previously (*22*).

Statistical analysis

Results are expressed as mean ± SD. Data were analysed using the unpaired two-tailed Student's *t*-test and the log-rank test. $P < 0.05$ were considered statistically significant.

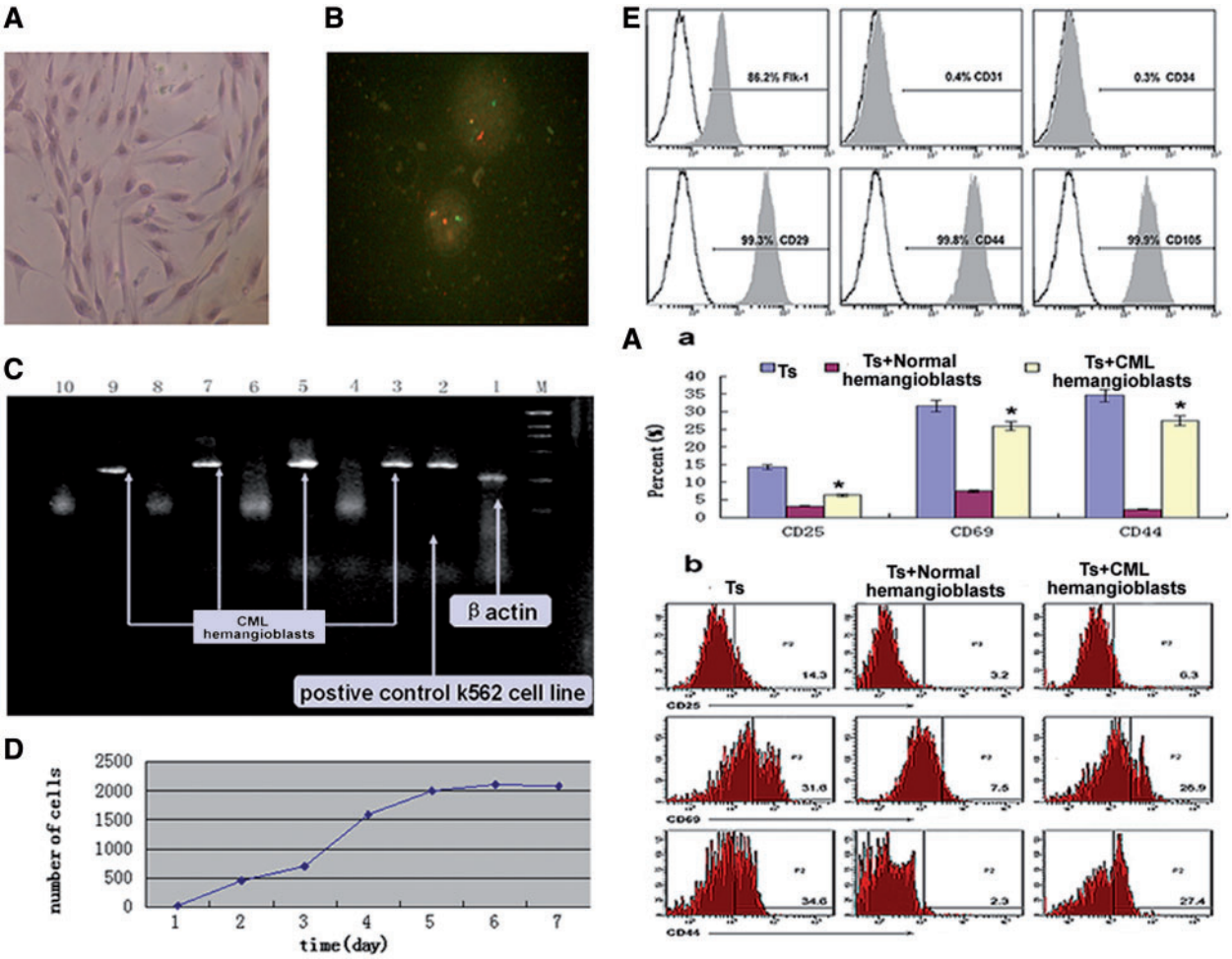

Fig. 1 Biological characteristics of the CML hemangioblasts. (A) The morphology of hemangioblasts from CML (magnification × 200). (B) BCR/ABL fusion gene was detected by FISH (yellow signal is the positive one) in CML hemangioblasts from male patients. (C) BCR/ABL fusion gene was detected by RT–PCR (lines 4, 6, 8, 10 correspond to non-special amplification). (D) The CML hemangioblasts proliferated with a population doubling time of ~23 h during the log phase of growth. (E) Isotype analysis showed they were all persistently negative for CD34 and CD31 but positive for Flk1, CD29, CD44 and CD105. (F) Comparison of the inhibitory effects of hemangioblasts on T lymphocyte activity. TS, PHA along; TS + CML hemangioblasts, CML hemangioblasts and PHA; TS+ hemangioblasts from healthy donors, healthy hemangioblasts and PHA. The proportion of hemangioblasts and T lymphocytes was 1 : 10, CD69, CD25 and CD44 were examined 12, 24 and 72 h later, respectively (columns represent percentage mean ± SD of three different experiments,* $P < 0.05$ versus Ts).

Results

The biological characteristics of CML hemangioblasts

To establish the characteristics of CML hemangioblasts, we first examined the morphology, phenotype and growth patterns of them respectively. Results showed that they persistently displayed fibroblast-like morphology (Fig. 1A) and proliferated with a population doubling time of ~23 h during the log phase of growth (Fig. 1D); besides, CML-specific BCR/ABL oncogene was observed by FISH analysis (Fig. 1B) and PCR (Fig. 1C) in CML hemangioblasts. Isotype analysis indicated they were all persistently negative for CD34 and CD31 but positive for Flk1, CD29, CD44 and CD105 (Fig. 1E). They could inhibit T lymphocyte activation and the percentage of CD25, CD69 and CD44 in PHA-induced T lymphocyte was 12.3 ± 3.5%, 34.5 ± 5.9% and 29.4 ± 7.0%, respectively, but they were 3.1 ± 2.3%, 6.4 ± 3.2% and 2.1 ± 1.7% when co-cultured with normal hemangioblasts and, when co-cultured with CML hemangioblasts, they were 5.4 ± 2.3%, 31.5 ± 6.8% and 24.5 ± 3.6%, respectively (Fig. 1F).

CML hemangioblasts promote recruitment of s-KitL and s-ICAM-1 upregulated by MMP-9

Further studies showed that MMP-9, s-KitL and s-ICAM-1 in CML hemangioblasts were higher than those in healthy donor hemangioblasts evidenced by western and PCR (Fig. 2A). The results indicated that CML hemangioblasts 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 CML hemangioblasts to deplete the corresponding mRNA and protein and this extinction did result in downregulation of s-KitL and s-ICAM-1 illustrated by western, PCR

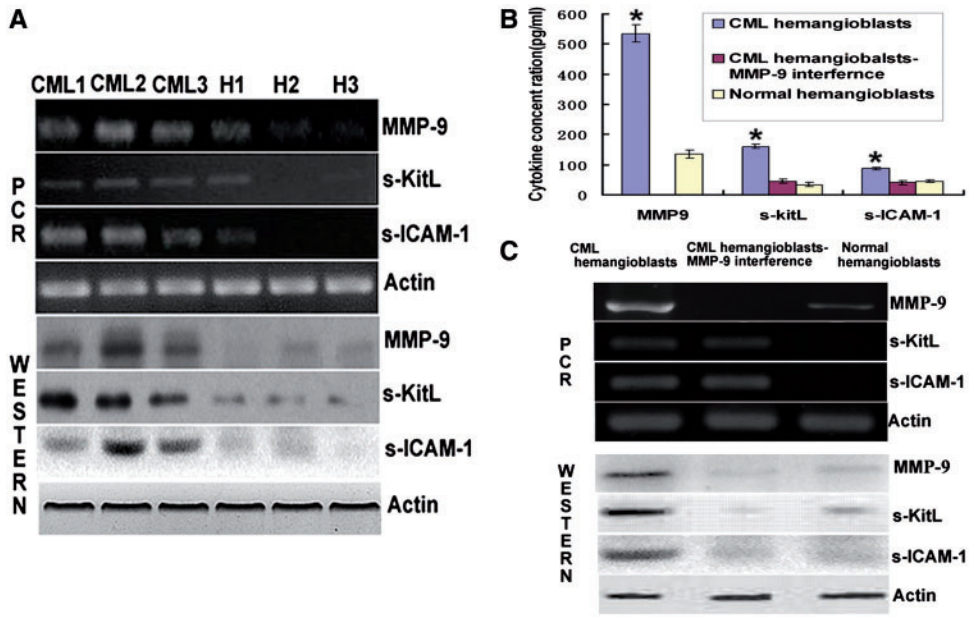

Fig. 2 MMP-9-mediated release of s-KitL and s-ICAM-1 in CML hemangioblasts. (A) Western blot and RT–PCR assayed the level of MMP-9, s-KitL and s-ICAM-1 of hemangioblasts from CML and healthy donors. (B) Hemangioblasts from CML, CML MMP-9(i) and healthy donors expressed different levels of MMP9, s-KitL and s-ICAM-1. Columns represent cytokine concentration mean ± SD of three different experiments, $^{*}P < 0.05$. (C) RT–PCR and western blot assayed the levels of MMP9, s-KitL and s-ICAM-1 of hemangioblasts from CML, CML-MMP-9(i) and healthy donors, respectively.

and enzyme-linked immunosorbent assay (ELISA) (Fig. 2B and C).

Abl kinase up-regulated TGF-β1 in CML

To investigate the mechanism involved in MMP-9 synthesis, we first assayed the expression of TGF-β in hemangioblasts from CML and healthy donors. As TGF-β has three isotype, we examined the expression of them. The results showed that TGF-β1 expression was apparently higher than TGF-β2 and TGF-β3 as shown in Fig. 3A and B. Thus, further research was aimed to TGF-β1. The effect of various doses of TGF-β1 on MMP-9 production by CML hemangioblasts was assessed to correlate the MMP-9 levels with the induction of signalling components including PI3K, Akt and NF-κB leading to the synthesis of the enzyme (Fig. 3C). Results from ELISA showed TGF-β1 up-regulated MMP-9 production both in hemangioblasts from CML and healthy donors (Fig. 3D). Those results demonstrated that BCR/ABL oncogene and TGF-β1 were involved in the regulation of MMP-9 production. We then examined the relationship of CML-specific BCR/ABL oncogene with TGF-β1, MMP-9, s-KitL and s-ICAM-1. Imatinib, inhibitor of BCR/ABL oncogene, blocked the induction of TGF-β1 and concomitantly MMP-9, s-KitL and s-ICAM-1 in a dose-dependent manner (Fig. 3E).

TGF-β1 up-regulates MMP-9 production via PI3K/Akt/NF-κB not Smad signalling pathway

To determine the involvement of PI3K signalling pathway in CML hemangioblasts, we initially examined the effect of PI3K activity on the induction of MMP-9 by TGF-β1. The addition of Wortmannin, inhibitor of PI3K, SH-5, inhibitor of Akt and SN-50, inhibitor of NF-κB, suppressed the MMP-9 synthesis induced by TGF-β1, respectively (Fig. 4A). We then analysed nuclear extraction by EMSA for DNA binding activity of NF-κB. As shown by Fig. 4B, lanes 1, 2 and 3 represented the results of nuclear extraction of CML hemangioblasts. CML hemangioblasts with 5 ng/ml TGF-β1 pre-culturing for 2 h and the results indicated that NF-κB was involved in TGF-β1 induced MMP-9 production. We at the same time analysed total cytoplasmic NF-κBp65 and phosphorylated NF-κBp65 in the presence or absence of TGF-β1 (5 ng/ml) treatment using western blot and ELISA (Fig. 4C) and the results showed that TGF-β1 up-regulated phosphorylated while NF-κBp65 had little effects on total NF-κBp65 production. As Smad was the main regulating pathway of TGF-β1, we then investigated whether Smad-regulated PI3K/Akt in CML hemangioblasts. As shown in Fig. 4D, Smad inhibitor HSc025 did not blocked the expression of MMP-9 induced by phosphorylated PI3K and Akt, indicating that PI3K regulated MMP-9 synthesis independent of Smad signalling pathway. Moreover, we further examined MMP-9 and phospho-SMAD2 induction by TGF-β1 in the presence of HSc025. The addition of HSc025, inhibitor of Smad, has no effect on MMP-9 synthesis induced by TGF-β1 and HSc025 actually worked in the experiment (Fig. 4E).

PI3K and Akt regulate NF-κB in CML hemangioblasts via IκB degradation

Previous assays indicted that Akt-regulated NF-κB expression through its activation with IKKα. IKKα was closely related with the phosphorylation of IκB, which was ubiquitinated and degraded by the proteasome

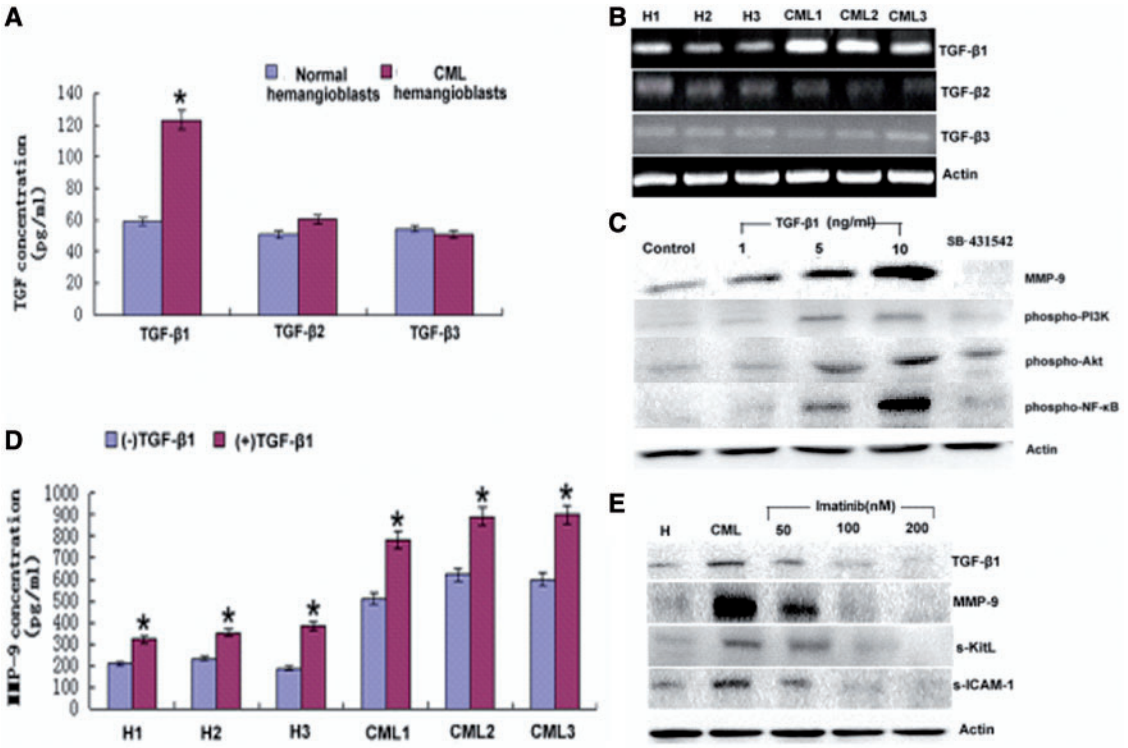

Fig. 3 BCR/ABL tyrosine kinase-induced TGF-β1 up-regulates MMP-9 in CML hemangioblasts. (A) The supernatants from BCR/ABL$^+$ hemangioblasts and healthy donor hemangioblasts were collected for ELISA to examine the expression of three isotypes of TGF-β and the results were representative of at least three independent experiments. *$P<0.05$. (B) RT–PCR results of three isotypes of TGF-β. The results were representative of at least three independent experiments. (C) BCR/ABL$^+$ hemangioblasts were treated with TGF-β1 (5 ng/ml) and SB-431542 (100 nM), then cell lysates were analysed by western blot with actin as the loading control. (D) BCR/ABL$^+$ hemangioblasts were treated with TGF-β1 (5 ng/ml) and supernatants were collected and MMP-9 was analysed by ELISA. Columns represent cytokine concentration mean ± SD of three different experiments, *$P<0.05$. (E) BCR/ABL$^+$ hemangioblasts were treated with imatinib for 2 h and then cell lysates were analysed by western blot.

complex. NF-κB was then activated and translocated to the nucleus (*23*). To determine whether Akt regulated NF-κB in CML in the same way, we first examined the expression of the three isotypes of IKK, IKKα, IKKβ and IKKγ on hemangioblasts from both CML and healthy donors. As evidenced by Fig. 5A, IKKα level was obviously higher in CML hemangioblasts. Next, CML hemangioblasts were treated with TGF-β1 for 2 h and then cell lysates were analysed by western blot with anti-IκB. The results showed that TGF-β1-suppressed IκB in a dose-dependent manner (Fig. 5C). Third, we treated CML hemangioblasts with Wortmannin, SH-5 and SN-50 for 1 h prior to the addition of TGF-β1 for 2 h and harvested cell lysates for immunoprecipitation to verify the relationship of phospho-Akt and phospho-IKKα and the results indicated that the phosphorylation of Akt and IKKα were blocked by Wortmannin and SH-5 but not SN-50 (Fig. 5B). Finally, we examined the degradation of IκB by western blot (Fig. 5D) and the activation of NF-κB p65 by EMSA (Fig. 5E) after the treatment of TGF-β1 in the absence or presence of the PI3K/Akt/NF-κB-specific inhibitors, respectively. The results indicated that elevated levels of TGF-β1 led to IκB degradation and activation of NF-κB p65. The fact that this process could be blocked by the inhibitors demonstrated that PI3K and Akt in the pathway led to the downstream activation of NF-κB in CML and subsequently up-regulated MMP-9 synthesis.

Discussion

CML is a clonal haematopoietic stem cell disorder characterized by the chromosome translocation and resultant production of the constitutively activated *BCR/ABL* tyrosine kinase (*25*). Although stem cell transplantation is the standard therapeutic option, transplant-related morbidity from graft-versus-host disease and mortality rates of 10–20% have greatly reduced the allogeneic haematopoietic cell transplantation in clinics (*26*), while interferon-α is only effective in some patients to some degree and chemotherapeutic intervention does not result in prolonged overall survival (*27*, *28*) and the reason is possibly due to some unknown biology of the CML cancer stem cells (*29*).

Our laboratory have identified the Flk-1$^+$ CD34$^-$CD31$^-$ hemangioblasts as the CML initiating cells and proved the rearrangement of the BCR/ABL gene might happen at the level of this hemangioblastic progenitor cells instead of HSCs. Based on this concept, we first examined the biological characteristics of CML hemangioblasts. FISH analysis of the Flk-1$^+$CD34$^-$CD31$^-$ hemangioblasts derived from CML bone marrow showed that they were BCR/ABL$^+$ which indicated that this oncogene had already

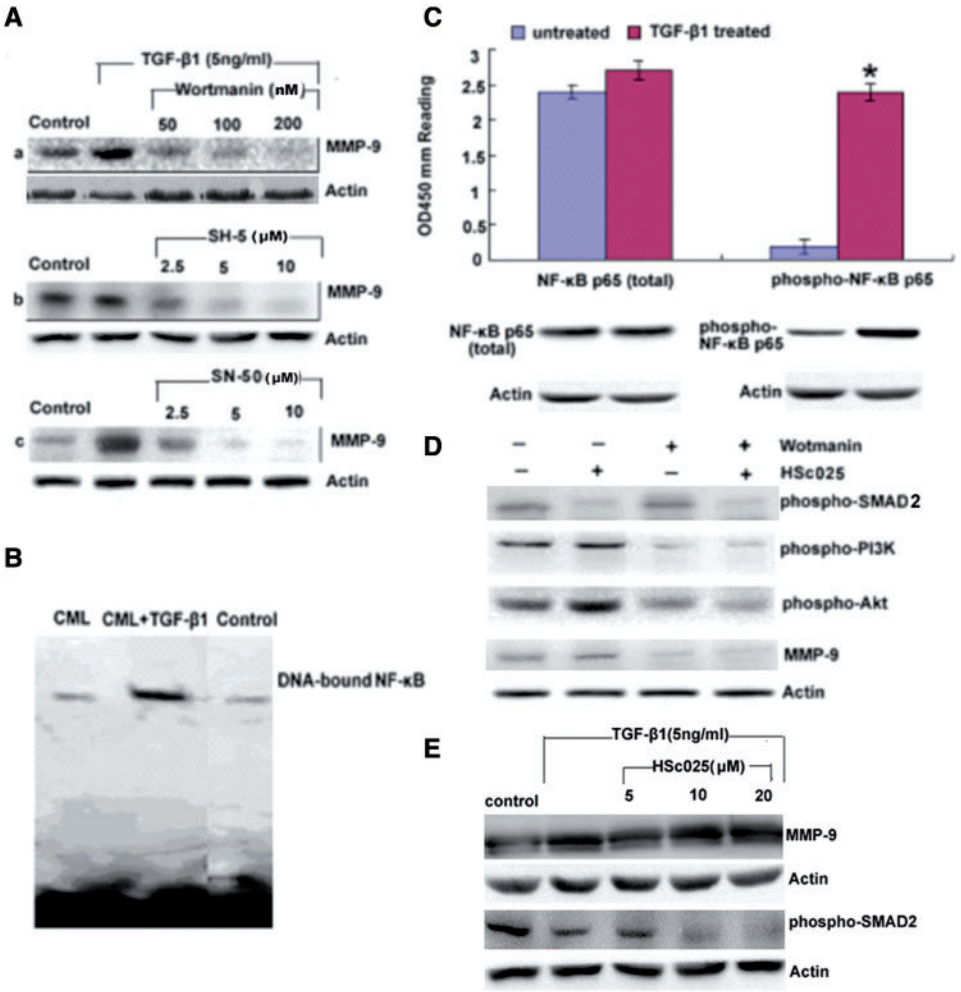

Fig. 4 TGF-β1 up-regulates MMP-9 production via PI3K/Akt/NF-κB, but independent of Smad signalling pathway. (A) BCR/ABL$^+$ hemangioblasts were treated with (a) Wortmannin (b) SH-5 and (c) SN-50 for 1 h prior to the addition of TGF-β1. Culture supernatants were harvested after 48 h and analysed for MMP-9 by western blot. Me_2SO 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) Total cell proteins was collected 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 is shown at the top and the corresponding western blot using phospho-NF-κBp65 (Ser536) rabbit mAb (right panel) or NF-κBp65 antibody (left panel) is shown in the bottom. Columns represent cytokine concentration mean ± SD of three different experiments, $*P < 0.05$. (D) Cell extracts from BCR/ABL$^+$ hemangioblasts treated with Wortmannin (100 nM) or HSc025 (5 μM) were examined for phosphorylated SMAD2, PI3K, Akt and MMP-9 by western blot. (E) BCR/ABL$^+$ hemangioblasts were treated with HSc025 for 1 h prior to the addition of TGF-β1. Culture supernatants were harvested after 48 h and analysed for MMP-9 and phospho-SMAD2 by western blot. Me_2SO was also added to one of the cultures as a control.

mutated at the hemangioblast level. More importantly, the ability of CML-derived hemangioblasts to inhibit T lymphocytes was weaker than that of normal hemangioblasts. Besides, a lot of blood disease such as acute lymphocytic leukaemia, chronic lymphocytic leukaemia and myelodysplastic syndrome are found to expressed high level of s-ICAM-1 and clinical researches indicated the level of it correlated directly with disease stage, prognosis and survival period (*30–32*). All these inspired us to further examine the s-KitL and s-ICAM-1, and both of them were higher in hemangioblasts from CML than those from normal donors. So, we believe that enhanced expression of s-ICAM-1 may facilitate tumor cells immune evasion by blocking the ICAM/LFA connection and prevent them from being recognized by T lymphocyte and NK cells. Increased s-KitL may also promote c-kit$^+$ HSCs recruitment and mobilization in CML, resulting in extramedullary leukemia cells infiltration. Besides, it further confirms our belief that purified true leukaemia stem cells could provide a target for immune-based therapies and biological response modifiers (*33*).

MMP-9 is important for maintaining normal hematopoietic process and 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 influence cells recruitment and differentiation (*34–36*). In the present investigation, we found that CML hemangioblasts expressed high level of MMP-9. We also found that the s-KitL and s-ICAM-1 was MMP-9 dependent. To further investigate the mechanism behind MMP-9 production in BCR/ABL$^+$ hemangioblasts, we examined the expression of the three

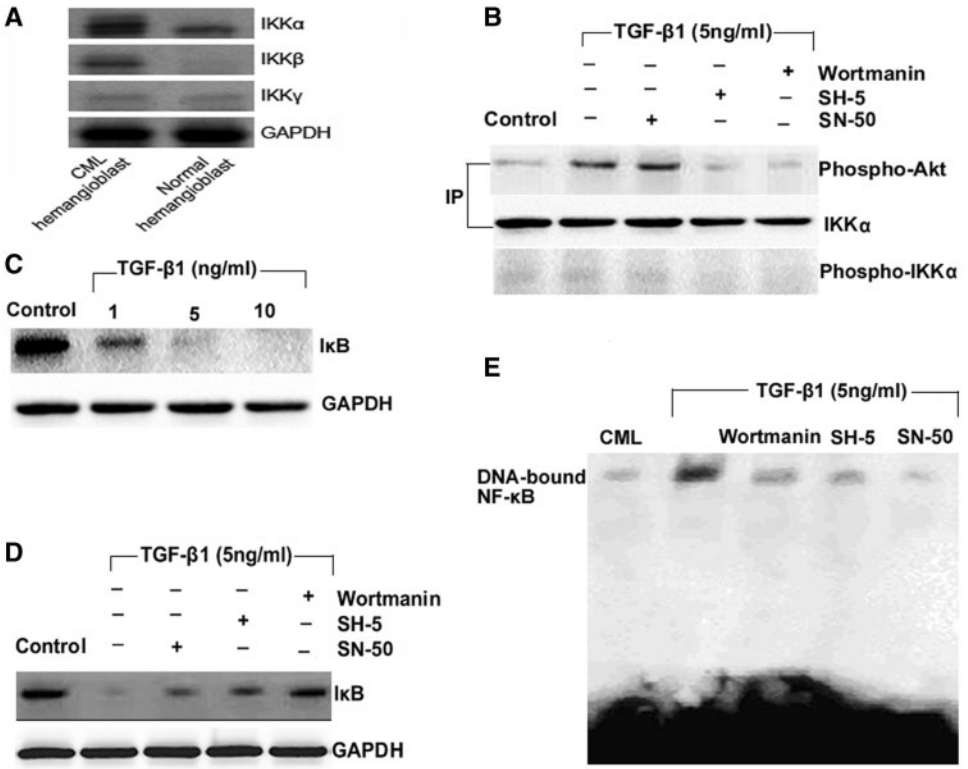

Fig. 5 PI3K/Akt/NF-κB signalling pathway regulates TGF-β1 in BCR/ABL$^+$ hemangioblasts. (A) Cell extracts (60 μg protein) from BCR/ABL$^+$ hemangioblasts and healthy donors were analysed by western blot with anti-IKKα, anti-IKKβ and anti-IKKγ, and GAPDH was used as the loading control. (B) BCR/ABL$^+$ hemangioblasts were treated with Wortmannin (100 nM), SH-5 (5 μM) and SN-50 (5 μM) for 1 h, prior to the addition of TGF-β1 (5 ng/ml) for 2 h. Cell lysates were prepared and immunoprecipitated with anti-IKKα and then analysed by western blot with anti-phospho-Akt or anti-phospho-IKKα. (C) BCR/ABL$^+$ hemangioblasts were treated with TGF-β1 for 2 h and then cell lysates were analysed by western blot with anti-IκB and GAPDH as the loading control. (D) BCR/ABL$^+$ hemangioblasts were treated with Wortmannin (100 nM), SN-50 (5 μM)and SH-5 (5 μM)for 1 h, prior to the addition of TGF-β1 (5 ng/ml) for 2 h and then cell lysates were prepared and analysed by western blot with anti-IκB or anti-GAPDH. (E) BCR/ABL$^+$ hemangioblasts were treated with Wortmannin (100 nM), SN-50 (5 μM) and SH-5 (5 μM) for 1 h, prior to the addition of TGF-β1 (5 ng/ml) for 2 h and then nuclear extracts were harvested to examine the NF-κB binding ability by EMSA.

isotypes of TGFβ and found TGF-β1 was abnormally higher, which upregulated MMP-9 in a dose dependent manner. Thereby, in the next research, we focused on the relationship between BCR/ABL oncogene and TGF-β1. We found that imatinib, inhibitor of BCR/ABL oncogene, blocked the induction of TGF-β1 in a dose-dependent manner. This indicated that this BCR/ABL oncogene up-regulated TGF-β1 in CML. Møller (*37*) showed that the growth of Bcr-Abl-expressing CD34+ cells from chronic phase CML patients is inhibited by TGF-β and, interestingly, treatment of a non-proliferating CD34+ CML cell sub-population with the TGF-β kinase inhibitor SB431542 enhanced cell death mediated by the Bcr-Abl inhibitor imatinib. At present, little is known regarding how TGF-β influence CML cell proliferation in the context of Bcr-Abl expression. We hypothesize that there might exist certain novel cross-regulatory mechanism in maintaining the transformed progenitor cell population and we further showed that TGF-β1 might play a central role in CML pathogenesis. Actually, BCR/ABL and TGF-β was proved to be involved in the cell function by other reports (*38–40*).

Further evidence shows that imatinib also blocked s-KitL and s-ICAM-1 in a dose-dependent manner. An important downstream signalling molecule of TGF-β1 is Smad. There are also Smad-independent pathway, such as Ras (*41*), Rho proteins (*42*), extracellular signalling kinase (*43*) and p38 (*44*). Recently, phosphatidylinositol 3-kinase (PI3K) is found to be involved in TGF-β1 signalling positively or negatively at least in some cell types as both stimulation and inhibition effects have been described (*45–47*). To investigate whether TGF-β1 was Smad dependent or independent in BCR/ABL$^+$ hemangioblasts, we used Smad and PI3K inhibitor, respectively as indicated and the results showed that it depended on PI3K instead of Smad. Actually, TGF-β activated PI3K has been reported in many cell lines (*48*, *49*).

It was reported that PI3K was activated by tyrosine kinase (*50*, *51*) and our results further verified that in CML, it was activated by tyrosine kinase-induced TGF-β1. Following its recruitment to these receptors in the plasma membrane, PI3K is activated and phosphorylated 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 (*52*). When the capability of PI3K to produce PIP3 was blocked in BCR/ABL$^+$ hemangioblasts 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$^+$ hemangioblasts, primarily through Akt and not other downstream components of PI3K containing a PH domain. SN-50 inhibition of MMP-9 production indicated that NFκB was also involved in this pathway which coordinated with the reports of other cell lines (*22*).

Three members of the IKK family have been isolated, and these are now referred to as IKK1/IKKα, IKK2/IKKβ and IKK3/IKKγ. Expression and the ratio of IKKα to IKKβ vary among cell types. NF-κB, in the cells with a high proportion of IKKα to IKKβ, is sensitive to Akt activity (*53*). Our data showed that IKKα was predominant in human BCR/ABL$^+$ hemangioblasts, which was apparently different from that in healthy donors where the three subunits existed at similar level. Based on these data, we hypothesized that Akt might bind to IKKα constitutively and their phosphorylation might occur following TGF-β1 stimulation. Immnoprecipitation confirmed our idea which showed that the phosphorylation of Akt was regulated by PI3K and Akt subsequently phosphorylated IKKα which regulated the activation of NF-κB.

Finally, IκB proteins are degraded rapidly by the proteasome complex once ubiquitinated, thereby freeing NF-κB, which then enters the nucleus, binds to DNA, and activates transcription (*54*). It had been shown that Akt regulated NF-κB activation directly through the activation of IKKα and our findings demonstrated that the PI3K/Akt pathway also regulated IκB degradation and activation of NF-κB in BCR/ABL$^+$ hemangioblasts stimulated by TGF-β1.

Generally speaking, a intriguing aspect of this study is the illustration of CML hemangioblast-HSCs being amplified by membrane-anchored cytokines which are mediated by CML-specific BCR/ABL oncogene induced signalling pathway. Modulation of local cytokines relating to HSCs fates provides promising hope for clinical targeting therapy.

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Conflict of interest

None declared.

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