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·*Original Article*·

Leukemic stromal hematopoietic microenvironment negatively regulates the normal hematopoiesis in mouse model of leukemia

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[**Abstract**] **Background and Objective:** Leukemic microenvironment has a major role in the progression of leukemia. Leukemic cells can induce reversible changes in microenvironmental components, especially the stromal function which results in improved growth conditions for maintaining the malignant leukemic cells. This study aimed to investigate the survival advantage of leukemic cells over normal hematopoietic cells in stromal microenvironment in long term. **Methods:** The mice were injected intraperitoneally with N-N' ethylnitrosourea (ENU) to induce leukemia; the mice received injection of normal saline were used as control. At 180 days after ENU induction, the mice were killed and the bone marrows were cultured for 19 days. Colony-forming assays were used to analyze the formation of various cell colonies. The expression of Sca-1, CD146, VEGFR2, CD95, pStat3, pStat5, and Bcl-xL in marrow cells were detected by flow cytometry. **Results:** Long-term leukemic bone marrow culture showed abnormal elongated stromal fibroblasts with almost absence of normal hematopoietic cells. Adherent cell colonies were increased, but CFU-F and other hematopoietic cell colonies were significantly decreased in leukemia group ($P < 0.001$). Primitive progenitor-specific Sca-1 receptor expression was decreased with subsequent increased expression of CD146 and VEGFR-2 in leukemic bone marrow cells. Decreased Fas antigen expression with increased intracellular pStat3, pStat5 and Bcl-xL proteins were observed in leukemic bone marrow cells. **Conclusions:** Stromal microenvironment shows altered morphology and decreased maturation in leukemia. Effective progenitor cells are decreased in leukemia with increased leukemia-specific cell population. Leukemic microenvironment plays a role in promoting and maintaining the leukemic cell proliferation and survivability in long term.

Key words: Stromal microenvironment, hematopoiesis, leukemia

Hematopoiesis is a dynamic process resulting in continuous production of mature blood-forming cells from a relatively small population of primitive hematopoietic stem/progenitor cells through diverse proliferative and differentiative events [1-4]. Recent studies have revealed that the normal hematopoiesis requires associative interaction between hematopoietic cells and the cells of the hematopoietic microenvironment which are necessary for the critical maintenance of cell proliferation, maturation, and death [5-10]. Disruption of this fine balance between cell proliferation, cell survival and cell death plays a major role in development of several neoplastic diseases, particularly leukemia. Leukemia is a stem cell disease where normal stem cell function and behavior are lost due to transformation events [11,12]. Leukemia development is a multi-step process characterized by progressive cellular transformation of normal hematopoietic stem/progenitor cells into malignant leukemic clones. These leukemic clones could induce reversible changes in stromal function or composition which results in improved growth conditions for malignant cells (malignancy-induced microenvironment). The functionally abnormal malignant clones become an integral

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part of the stromal system, selectively stimulating neoplastic cells and inhibiting normal blood cell formation (malignant microenvironment) [13,14]. Although malignant cells have an intrinsic growth and survival advantage, but they also remain dependent on some factors provided by the malignant microenvironment. Several recent studies have found the existence of a leukemic microenvironment [15], however, the exact scenario of the alteration in stromal hematopoietic microenvironment and stroma-dependent surveillance of malignant cells still remains unclear.

In this study, we induced leukemia in mice by a potent carcinogenic agent N-N' ethylnitrosourea (ENU). The initiation and progression of leukemia were ascertained by analyzing the blood hemogram profile and peripheral blood smears. The alteration in stromal hematopoietic microenvironment in leukemia was assessed by long-term bone marrow cultures. Bone marrow adherent cell colony formation and colony-forming unit-fibroblast (CFU-F) assay were done to analyze the functional alteration of stromal hematopoietic components in leukemia if any. Further, the hematopoietic progenitor cell colony assays were used to analyze the stroma-associated changes in functional maturation or terminal differentiation of hematopoietic progenitor cells in leukemia. The expression of Sca1, CD146, vascular endothelial growth factor receptor 2 (VEGFR2), pSTAT-3, pSTAT-5, Bcl-xL, and CD95 in leukemic bone marrow cells were detected. Our data collectively hinted the role of leukemic microenvironment in maintaining leukemic cell survivability.

Materials and Methods

Animals and leukemia induction

A total of 120 inbred Swiss albino mice of either sex, aging 10–14 days, were obtained from the Institutional animal house. Sixty mice were injected intraperitoneally with ENU (Sigma Co., USA) at a dose rate of 80–100 mg/kg body weight. The mice were observed for 4 to 8 months. Peripheral blood hemogram was done to assess leukemic disease progression. Sixty mice received intraperitoneal injection of normal saline and were used as normal controls. During experiment, all mice were on normal diet and clean water ad libitum at 28℃.

Blood hemogram profile

At 180 days after ENU induction, about 200–300 μL of blood was collected from each mouse by tail-vein puncture to evaluate certain hematological parameters. White blood cell (WBC) count, reticulocyte count, and percentage of blast cells from differential WBC count were determined using standard laboratory techniques. Peripheral blood smears were prepared and stained with standard Leishman staining.

Isolation of bone marrow

At 180 days after ENU induction, the mice which showed progressive disease statuses were sacrificed to isolate the long bones (femurs and tibia). The long bones were cut in both ends by scissors and the red-pulp region of the marrow was flushed out with RPMI-1640 media (Sigma Co., USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, BRL). Some portions of the isolated marrow were cut into small pieces (about 0.2 mm^3) and kept in RPMI-1640 media containing 10% FBS for further culture protocol. The rest of the bone marrow was mixed well with repeated pipetting and passed through cell strainer to isolate single cells and washed in phosphate buffered saline (PBS) and finally transferred to RPMI-1640 media (without FBS). RBCs were then depleted using RBC-depletion buffer (BD Biosciences, USA). Cells were kept in ice until use.

Long-term bone marrow culture

The small pieces (0.2 mm^3) of bone marrow from each mouse were cultured in triplicate in 75-mm culture dish (Corning, USA) containing 4 mL of RPMI-1640 supplemented with 30% FBS, 1% bovine serum albumin (BSA), 0.02% (*V/V*) 2-mercaptoethanol, and 100 ng/mL recombinant mouse stem cell factor (SCF) (E-Biosciences, USA), 50 ng/mL recombinant mouse interleukin-3 (IL-3) (BD-Biosciences, USA), and 50 ng/mL recombinant mouse granulocyte-macrophage colony stimulating factor (GM-CSF) (BD-Biosciences, USA). The cells were cultured at 37℃ in an atmosphere of 5% CO_2 for 19 days. At different time course, the culture plates were monitored, and cellular growth pattern was observed and photographed under the inverted microscope.

Adherent cell colony assay

For the bone marrow adherent cell colony assay, bone marrow-derived cells were suspended in 4 mL of RPMI-1640 medium supplemented with 30% FBS, 100 u/mL penicillin, 100 u/mL streptomycin, and 0.02% (*V/V*) 2-mercaptoethanol at a concentration of 4×10^6 cells/plate in 75 mm × 10 mm culture plates at 37℃ and 5% CO_2. Cells from each mouse were cultured in four plates. At every 72 h interval, the media was drained off, and fresh media supplemented with 30% FBS and 0.01% (*V/V*) 2-mercaptoethanol was added for the maintenance of the culture. After 16 days of culture, the numbers of adherent colonies were scored using an inverted microscope.

Colony-forming unit-fibroblast (CFU-F) assay

About 1–1.5 × 10^6 bone marrow-derived cells were plated into 75-mm culture plate (Corning, USA) in triplicate in α-MEM with 30% FBS, L-glutamine, 100 u/mL streptomycin, 100 U/mL penicillin, and 0.01% (*V*/*V*) 2-mercaptoethanol. At every 3rd day, the media was drained off and new media was added. For 2 weeks, the cultures were maintained at 37℃ and 5% CO_2. The resulting colonies were then scored using an inverted microscope.

Hematopoietic progenitor cell colony forming assay

Hematopoietic progenitor cells were assayed in methylcellulose-based semisolid media. The culture media consisted of 1.2% methylcellulose, 30% FBS, 1% BSA, 0.02% (*V*/*V*) 2-mercaptoethanol, and 100 ng/mL recombinant mouse SCF, 50 ng/mL recombinant mouse IL-3, and 50 ng/mL recombinant mouse GM-CSF. RBC-depleted bone marrow cells were plated at a final concentration of 5 × 10^5 cells/mL in triplicate and cultured at 37℃ in 5% CO_2. After 16 days of culture, colonies were scored using an inverted microscope.

Flow cytometric analyses of surface receptors expression

RBC-depleted bone marrow cells were washed and fixed with 3% paraformaldehyde (PFA). To detect the membrane bound expression of Sca1, CD146, VEGFR2, and CD95, PFA-fixed bone marrow cells from each groups were put in 4 tubes (1 × 10^6 cells /tube) and treated with 2 μL of anti-mouse Sca1 PE-, anti-mouse CD146 FITC-, anti-mouse VEGFR2 PE- and anti-mouse CD95 PE-conjugated monoclonal antibodies, then incubated for 30 min in dark at 37℃. Finally, the cells were washed in PBS to wash off the excess fluorescence. Each tube was analyzed by a BD-FACS Calibur (Becton Dickenson, San Jose, CA) according to the standard procedures and the data were analyzed using CellQuest pro software.

Flow cytometric analyses of intracellular pSTAT-3, pSTAT-5, and Bcl-xL

The bone marrow cells were initially incubated in RPMI-1640 media containing 30% FBS with the combination of supportive microenvironmental factors like IL-3 (50 ng/mL), GM-CSF (50 ng/mL), and SCF (100 ng/mL), respectively, for 4 h at 37℃. To detect the intracellular expression of pSTAT-3, pSTAT-5, and Bcl-xL, about 3 × 10^6 stimulated bone marrow were mixed with 1.5% paraformaldehyde, incubated in the fixative for 10 min at room temperature, and pelleted and washed thrice with PBS, then permeabilized by resuspending with vigorous vortexing in 500 μL ice-cold methanol per 1 × 10^6 cells and incubated at 4℃ for 15 to 20 min. Cells were then washed twice in PBS containing 1% BSA and divided in 3 sorting tubes; each one contained 1 × 10^6 cells in 100 μL media. Then 2 μL of rabbit anti-mouse pStat-3, pStat-5, and Bcl-xL primary mAbs (Cell Signaling Technologies, USA) were added to the respective labeled tube and incubated for 30 min at room temperature followed by the addition of goat anti-rabbit secondary antibody conjugated with Alexa Fluor-488 (Invitrogen, USA) to tube and incubated further for 30 min at room temperature. The cells were then washed with 15 volumes of staining media and pelleted. Finally, the pellets were resuspended in respective tubes containing 400 μL of staining media and analyzed by BD FACS Calibur (Becton-Dickenson, USA), using cell quest pro software.

Statistical analysis

All data are presented as mean ± standard deviation. Student's *t* test was performed for intergroup comparison. A probability of $P < 0.01$ was considered to be statistically significant.

Results

Leukemia development in mice

Leukemia development was verified through peripheral blood hemogram profile and peripheral blood smear analysis. Of the 60 ENU-treated mice, 36 showed progressive leukemic disease status. To maintain parity in our study, we continued our experiments with 36 normal control mice.

Peripheral blood hemogram profile

Peripheral blood hemogram profiles showed that, compared with those in control mice, WBC count and the proportion of reticulocytes were significantly increased in ENU-induced leukemic mice ((36 ± 2.57) × 10^3/μL vs. (6.4 ± 1.28) × 10^3/μL, $P < 0.01$; (1.6 ± 0.09)% vs. (0.82 ± 0.15)% , $P < 0.01$), the proportion of neutrophils was decreased ((16.75 ± 2.19)% vs. (23 ± 2.24)%, $P < 0.01$), and the proportion of percentage of immature blast cells was increased ((35.10 ± 6.67)% vs. 0, $P < 0.01$). Observed under microscope, peripheral blood smear from leukemic

mice showed frequent abundance of blast cells of both lymphoid and myeloid lineages (Figure 1).

Long-term bone marrow culture

During the long-term culture, the bone marrow from control group showed no changes in the proportions and shapes of hematopoietic cells and stromal fibroblasts, whereas that from leukemic group showed an increase and elongation of stromal fibroblasts, resulting in a predominance and confluence of stromal fibroblasts (Figure 2).

Cell colony-forming assays

Adherent bone marrow cell colonies were significantly increased in leukemic group than in control group during the culture ($P < 0.01$), whereas fibroblast colony-forming efficacy was significantly decreased in leukemia group than in control group ($P < 0.001$) (Table 1).

Leukemic marrow hematopoietic progenitors showed an overall decrease in colony formation than normal hematopoietic progenitors. Granulocyte colony-forming units (CFU-G), granulocytes/macrophages colony-forming units (CFU-GM), granulocyte/erythrocyte/monocyte/macrophage colony-forming units (CFU-GEMM), and erythroid burst-forming units (BFU-E) were all significantly decreased in leukemia group than in control group ($P < 0.001$) (Table 1).

Table 1 The numbers of the bone marrow adherent and progenitor cell colonies from the mice in control and leukemic groups after 16 days of culture

Colony type	Control (mean ±SD)	Leukemia (mean ± SD)
Adherent cell colony	22 ± 1	30 ± 2
CFU-F	24 ± 2	13 ± 1
CFU-G	25 ± 1	18 ± 1
CFU-GM	29 ± 1	24 ± 1
CFU-GEMM	28 ± 2	18 ± 2
BFU-E	33 ± 1	14 ± 2

CFU-F, fibroblast colony-forming units; CFU-G, granulocyte colony-forming units; CFU-GM, granulocytes/macrophage colony-forming units; CFU-GEMM, granulocyte/erythrocyte/monocyte/macrophage colony-forming units; BFU-E, erythroid burst-forming units. All data are presented as mean ± standard deviation (SD) from 15 mice in each group. For all comparisons between leukemic and control groups, $P < 0.001$.

Flow cytometric analysis

The granulocytic precursors/granulocytic population was significantly increased in leukemia group than in control group ($P < 0.01$), whereas the lymphocytic precursors/lymphocytic population was significantly decreased in leukemic group than in control group ($P < 0.01$) (Figure 3).

Phenotypic characterization of Sca1 receptor expression on bone marrow cells indicated down-regulated $Sca1^{bright}$ population (more primitive progenitors), and $Sca1^{dim}$ population (less primitive progenitors) in leukemia group as compared with control group ($P < 0.01$) (Figure 4).

Phenotypic characterization of CD146 and VEGFR2 expression on bone marrow cells showed up-regulated CD146 and VEGFR2 expression in leukemia group as compared with control group ($P < 0.01$) (Figure 5). Fas antigen expression was also down-regulated in leukemia group as compared with control group ($P < 0.01$) (Figure 6).

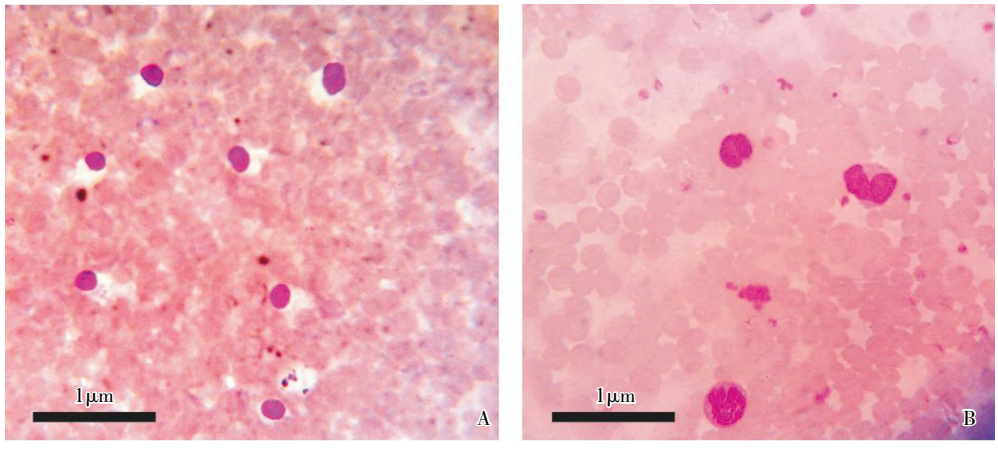

Figure 1 Peripheral blood smears from the control and leukemic groups of mice

The peripheral blood smears were stained with standard Leishman staining.

A, the mice in control group received intraperitoneal injection of normal saline. The peripheral blood smear showed normal cellular distribution pattern with no hypergranular blast cells. B, the mice in leukemia group received intraperitoneal injection of N-N' ethylnitrosourea (ENU) at a dose rate of 80-100 mg/kg body weight. The peripheral blood smear showed highly granular large cells, indicating they are immature, hypergranular blast cells.

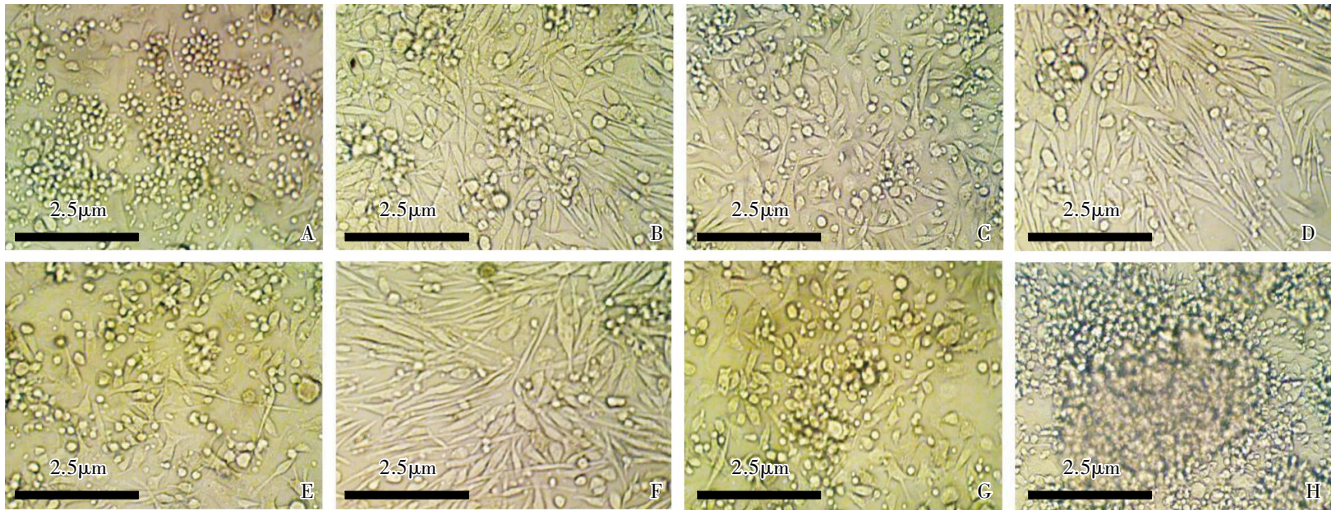

Figure 2 Long-term bone marrow cultures from mice in the normal and leukemic groups

A, at day 6 of culture, the bone marrow from mice in the control group showed a cumulative cell production and initiation of stromal matrix formation, with predominance of round hematopoietic cells over spindle-shaped stromal fibroblasts; B, at day 6, the bone marrow from mice in the leukemic group showed extensive generation of large stromal fibroblasts and less hematopoietic cells; C, at day 9, the bone marrow from control group showed an obvious balance between the production of hematopoietic cells and stromal fibroblasts with significant predominance of stromal fibroblasts; D, at day 9, the bone marrow from leukemic group generated stromal fibroblasts which were abnormally elongated with almost absence of hematopoietic cells; E, at day 14, the bone marrow from control group showed interdependent co-existence of hematopoietic cell colonies and stromal fibroblasts, which is essentially required for sustaining normal hematopoiesis; F, at day 14, the bone marrow from leukemic group showed that the highly elongated stromal fibroblasts reached a confluence and hematopoietic cells were kept aside; G, at day 19, the bone marrow from control group showed hematopoiesis with sufficient hematopoietic cell colonies upon the underlying adherent stromal microenvironment. The amount of torn-ended stromal fibroblasts significantly decreased; H, at day 19, the bone marrow from leukemic group showed highly increased leukemic bone marrow cells. The confluence could be observed on different parts of the previously created stromal microenvironment, but the concentration of stromal fibroblasts was significantly reduced.

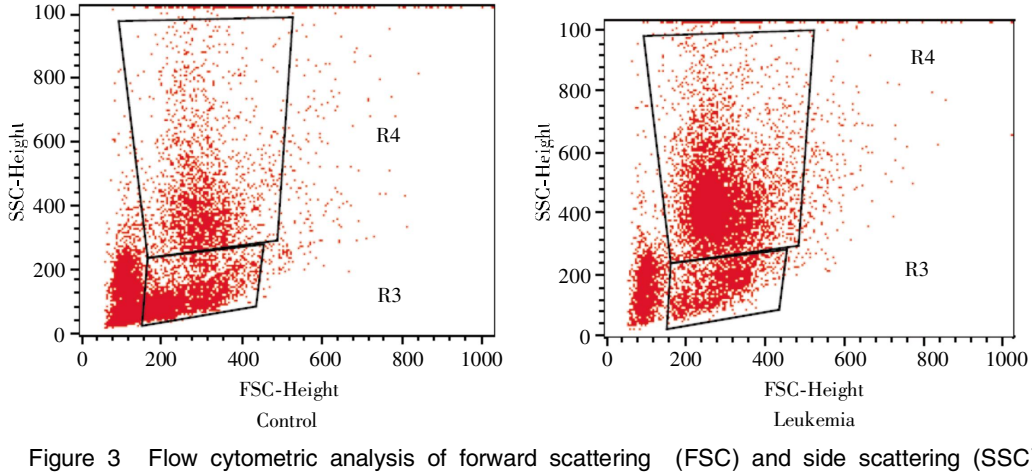

Figure 3 Flow cytometric analysis of forward scattering (FSC) and side scattering (SSC) characteristics of bone marrow cells from mice in leukemic and control groups

The R3 region denoted the lymphocytic precursors/lymphocytic population and the R4 region denoted the granulocytic precursors/granulocytic population. The proportion of lymphocytes in R3 region was significantly higher in control group than in leukemia group ((36.45 ± 1.43)% vs. (16.34 ± 0.82)%, $P < 0.001$); the proportion of granulocytes in the R4 region was significantly lower in control group than in leukemia group ((23.44 ± 1.42)% vs. (60.66 ± 2.34)%, $P < 0.001$).

The expression of pSTAT-3, pStat-5, and Bcl-xL in bone marrow cells were increased in leukemia group than in control group (Figure 7).

Discussion

Hematopoiesis is a complex process of several

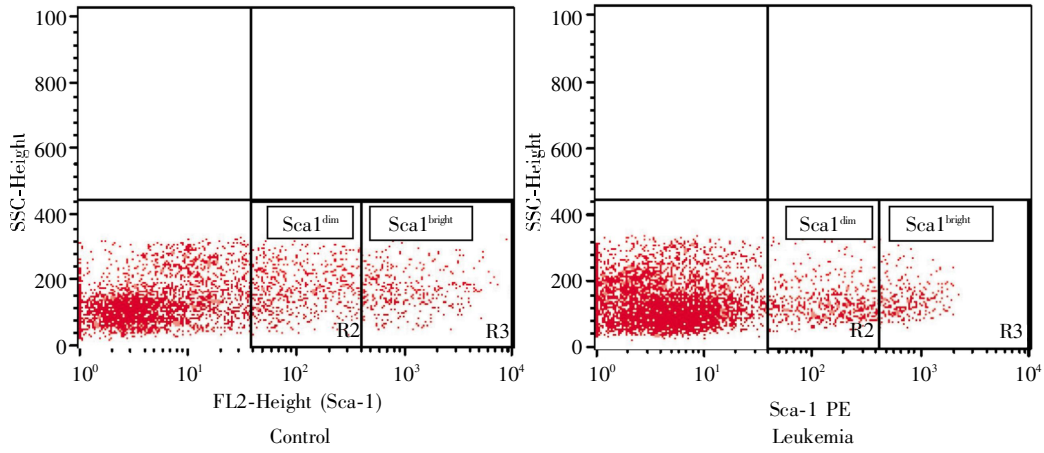

Figure 4 Phenotypic characterization of Sca1 receptor expression on bone marrow cells from mice in leukemic and control groups

Sca1-positive cells were decreased in leukemia group. $Sca1^{bright}$ population (more primitive progenitors in R3 region) and $Sca1^{dim}$ population (less primitive progenitors in R2 region) were decreased in leukemia group than in control group ((4.39 ± 1.10)% vs. (9.26 ± 0.99)%; (9.44 ± 0.81)% vs. (15.25 ± 1.2)%).

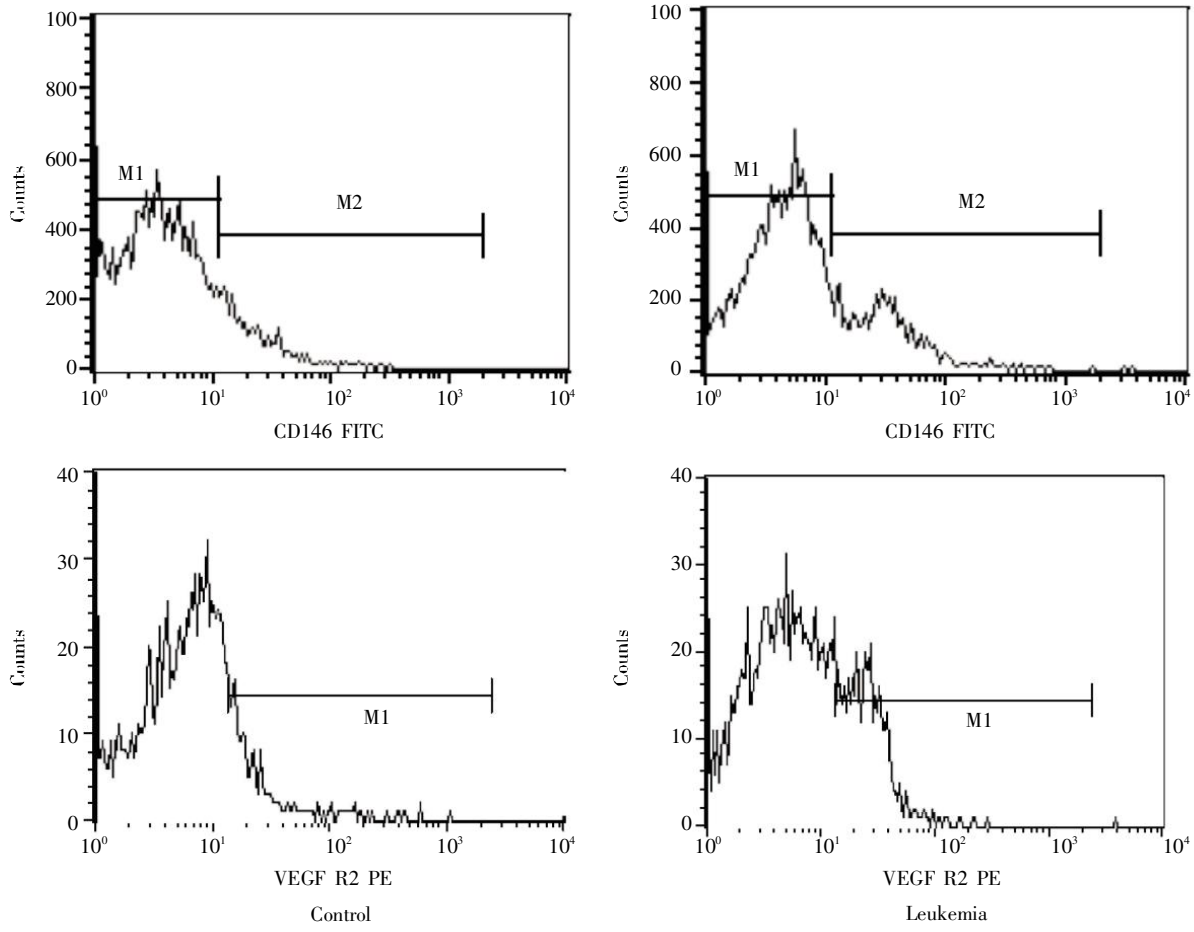

Figure 5 Phenotypic characterization of CD146 and VEGFR2 expression on bone marrow cells from mice in leukemic and control groups

CD146 expression and VEGFR-2 expression were significantly lower in control group than in leukemia group ((13.18 ± 0.51)% vs. (22.53 ± 1.45)%, $P < 0.01$; (12.96 ± 1.07)% vs. (26.68 ± 1.72)%, $P < 0.01$).

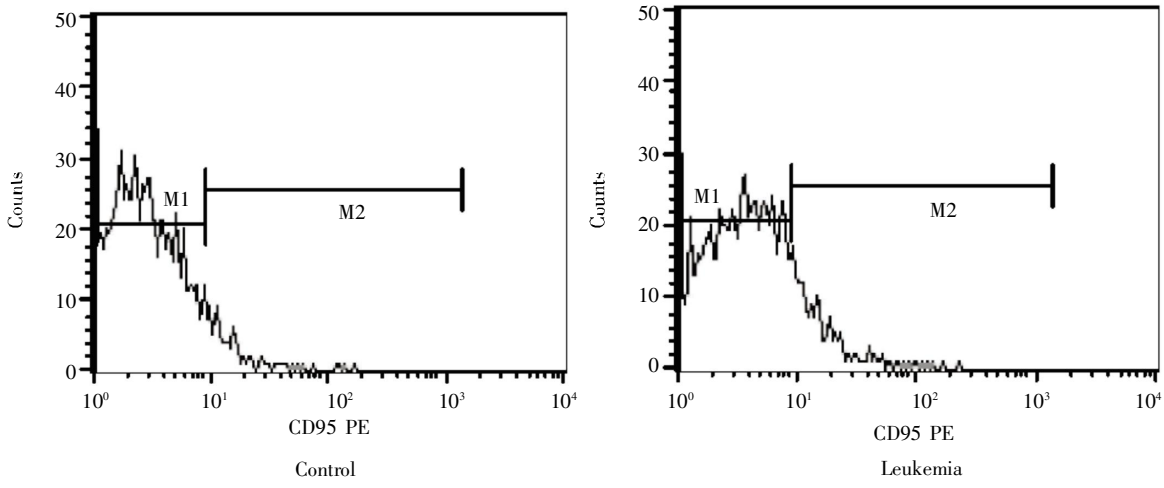

Figure 6 Phenotypic characterization of FasR (CD95) expression on bone marrow cells from mice in control and leukemic groups

FasR (CD95) expression was significantly higher in control group than in leukemia group ((5.66 ± 1.69)% vs. (2.39 ± 0.52)%, $P < 0.01$).

proliferative and differentiative signaling cascades responsible for the generation of healthy mature blood cells from a small population of hematopoietic stem/progenitor cells throughout the lifetime of an individual[16,17]. The blood cell formation from single stem/progenitor cells requires continuous stimulation of various cytokines and growth factors secreted from its surrounding microenvironmental accessory cells or the niche components [18-22]. Normal hematopoiesis is critically balanced by several cell proliferation, differentiation, and death signaling pathways. Alteration in one or more of these signaling pathways may ultimately shift the finely tuned equilibrium to other hypoproliferative or hyperproliferative disease condition. Under the condition of neoplastic progression such as leukemia, the normal hematopoietic cells can be transformed into leukemic cells. The resulting accumulative alterations in the leukemic cells shift the balance between cell proliferation and cell death towards the former, thus leading to a continuous increase of leukemic cells within the bone marrow microenvironment.

In this present study, we have used the mice model of leukemia developed by injecting ENU [23,24], a potent carcinogenic agent [25-28], to evaluate the changes in the characteristics of leukemia and deviation in the behavior of normal bone marrow microenvironment in leukemic disease progression. The disease progression was monitored by blood hemogram profile which demonstrated high increase in total granulocytic populations. The increased granulocytic population was confirmed by the flow cytometric analysis of leukemic bone marrow cells. The differential leukocyte count clearly showed significantly increased percentage of highly granular abnormal immature blast cells in the peripheral blood from mice in leukemic group.

We used the long-term bone marrow culture to assess the functional characterization of the stromal hematopoietic microenvironment and its role in aggravating the leukemic disease progression. A small piece of intact bone marrow (devoid of bone per se) can form the complex bone marrow microenvironment with appropriate architectural layout in in-vitro culture [29,30]. We used the technique of Friedenstein *et al.*[29] with some modifications to produce the entire meshwork of bone marrow stromal hematopoietic microenvironment from small pieces of intact bone marrow, to evaluate the changes in structure and function of the marrow stroma in leukemia. Our results clearly exhibited the defects in structure and function of the bone marrow stromal microenvironment during leukemogenesis. The bone marrow cells from control group could form the in-vivo hematopoietic stromal microenvironment in long-term culture condition. In normal bone marrow culture, the critical balance between adherent stromal matrix formation and hematopoietic cell generation was maintained during culture. In normal bone marrow cells, during the initial phase of the culture, the adherent stroma generation was rapid compared to hematopoietic cell development; however, the adherent stromal layer formation subsided with subsequent increased hematopoietic cell colony formation over the stromal adherent layer. In sharp contrast to the normal, the leukemic bone marrow cells showed almost complete shift of the equilibrium between adherent stromal layer formation and hematopoietic cell generation to the former. The leukemic bone marrow cells showed accelerated generation of abnormally elongated stromal cells in culture. The large stromal fibroblasts rapidly reached confluence in leukemia unlike the normal control. After the rapid formation of the complete meshwork of stromal hematopoietic

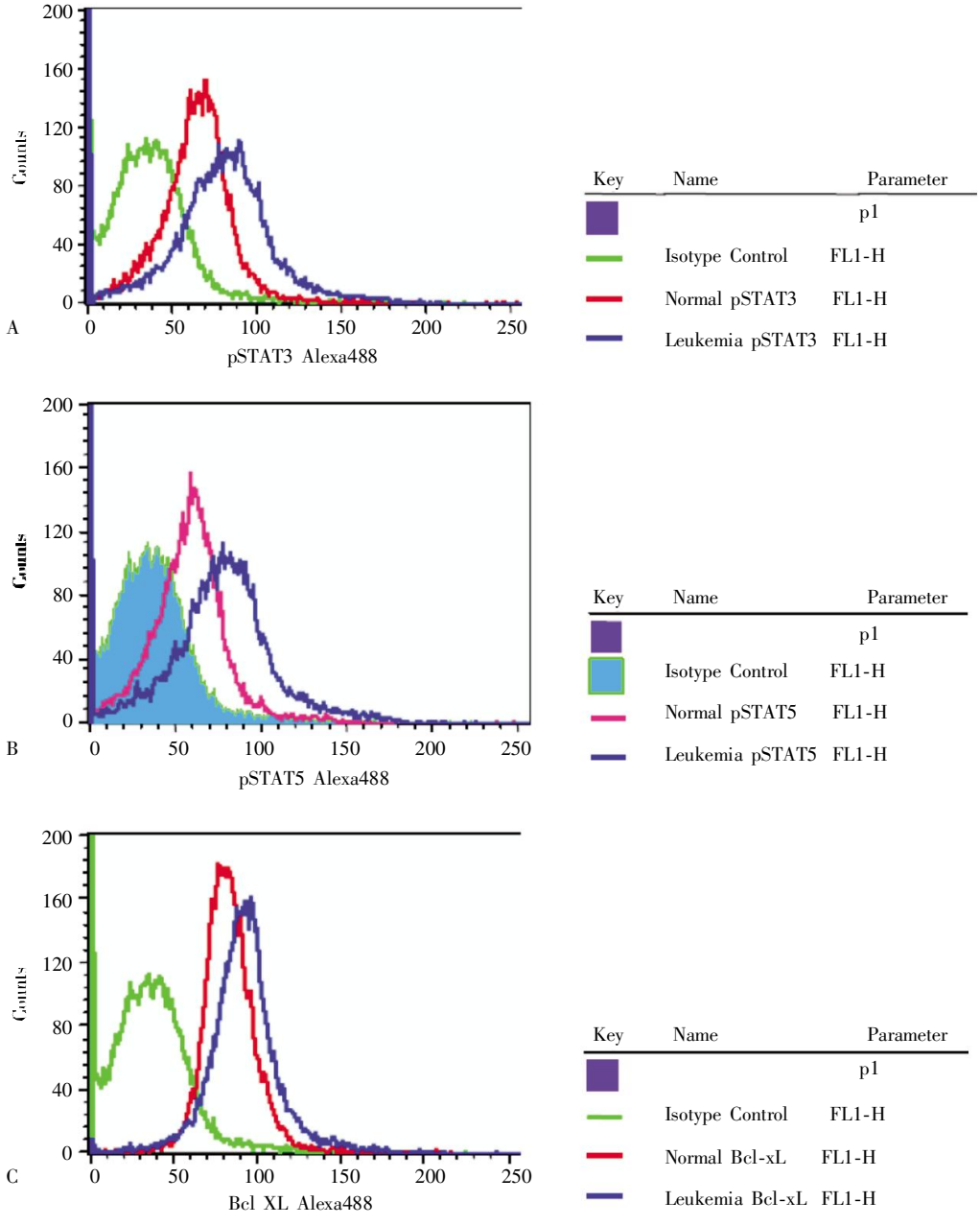

Figure 7 Flow cytometric analysis of pSTAT-3 (A), pSTAT-5 (B), and Bcl-xL (C) expression in bone marrow cells from mice in control and leukemic groups

The relative expression levels of pSTAT-3, pSTAT-5, and Bcl-xL are represented by fluorescence intensity along X-axis. The expression of pSTAT-3, pStat-5, and Bcl-xL in bone marrow cells were all increased in leukemia group than in control group (17.31 ± 0.84 vs. 9.53 ± 0.91, 15.70 ± 1.46 vs. 7.51 ± 0.87, 26.08 ± 0.95 vs. 16.22 ± 1.36, all $P < 0.01$).

microenvironment by the abnormal stromal components in leukemia, the stroma generations was subsided with increased development of giant confluent colonies upon it. The above mentioned phenomena could be explained by that the initial generation of the abnormal stromal layer only provided support and produced several survival factors for the increased need of the leukemic cells. Therefore, we speculate that the alteration of stromal hematopoietic microenvironment in leukemia could not only induce reversible changes in normal hematopoietic cells but also support the leukemic hematopoiesis in long term by hindering the normal hematopoietic event. The altered

microenvironment in leukemia seemed to have developed dynamic interactions between leukemic cells and the marrow microenvironment and provided a sanctuary for subpopulations of leukemic cells to act as a safeguard against conventional chemotherapy. Colony-forming ability of the bone marrow adherent cell population was moderately increased in leukemic group compared to control group. The moderately increased adherent cell colonies specifically hinted at the increased proliferation potential and adhesion property of leukemic bone marrow cells. The increased proliferation of leukemic bone marrow cells more or less maintained a reversible equation with the adherent cell colony numbers in leukemia. However, the functional maturation of the adherent population could not be extrapolated with the adherent cell colony formation in leukemia. The leukemic CFU-F numbers was decreased compared to the normal CFU-F numbers. This was directly related with the functional maturation / terminal differentiation of the stromal precursors which was significantly affected in leukemia despite of their high rate of proliferation. The decrease in hematopoietic cell colony formation (CFU-G, CFU-GM, CFU-GEMM, and BFU-E) essentially indicated the leukemic stroma-dependent suppression of normal hematopoiesis. Stromal cells developed in long-term bone marrow cultures of the leukemic group were deficient in enhancing erythropoiesis, granulopoiesis, and sustaining normal hematopoiesis. Our results suggested that the leukemic stroma provided a competitive growth advantage to the leukemic cells over the normal hematopoietic cells by inhibiting the functional maturation of the normal blood cells at the level of hematopoietic stem / progenitor cells.

Decreased Sca-1 receptor expression on leukemic bone marrow cells hints at the decreased primitive hematopoietic stem cells and suppression of normal hematopoiesis in leukemia. Recent studies suggest that the circulating endothelial cells derived from endothelial progenitor cells of the bone marrow and blood vessel endothelium of adults could participate in new blood vessel formation in normal and pathologic states including tumor progression [31-34]. Several reports have stated the increased expression of the adhesion molecule CD146 on endothelial cells in different tissue-specific tumor progression. We observed increased expression of CD146 on leukemic bone marrow cells. The increased CD146 expression highly correlates with the increased adhesion property of the bone marrow endothelial cells which bind leukemic cells to promote leukemogenesis. We also observed increased VEGFR2 expression in leukemia. Because the leukemic blasts and other cellular components of stromal microenvironment like stromal fibroblasts and stromal endothelial cells account for increased VEGFR2 expression [35], the increased endothelial cells in turn enhance the proliferation and support the maintenance of leukemic cells.

The complex interplay between leukemic cells and leukemic microenvironment alters several intertwined signaling processes involving cell growth, cell proliferation, and cell death that play vital roles in maintaining leukemic hematopoiesis [36]. The contribution of individual Stat proteins to normal cytokine signaling and development has been studied in various cell culture systems and in mice by gene disruption [37]. Some related roles and many unique non-overlapping physiological roles of various members of Stat family have been identified. Beyond these various roles in normal cellular and physiological processes, the STAT proteins also participate in cellular transformation and oncogenesis [38]. Subsequent work showed that in several hematological malignancies, the STATs, particularly STAT-3, are required to maintain the transformed phenotype of the cells [38]. STAT-5 is activated in certain hematological malignancies, especially leukemia. Recent reports have stated the role of STAT-5 in maintaining normal and leukemic hematopoiesis [39,40]. Our results also showed high expression of both pSTAT-3 and pSTAT-5 in the leukemic bone marrow cells. Some reports stated that STAT-3 activation is not essential for viability of normal and malignant cells [38]. Based on our data, we speculate that the expression of both pSTAT-3 and pSTAT-5 is essential for maintaining leukemic hematopoiesis and leukemic cell survivability in long-term culture. The evasion of programmed cell death (PCD) attracts much attention in cellular physiology that dictates the growth of malignant cells and is a hallmark of all cancers [41,42]. Our results also demonstrated an anti-apoptotic behavior of leukemic cells by up-regulating the expression of anti-apoptotic protein Bcl-xL, a Bcl-2 family protein which is known to be a key regulator of apoptosis [43,44]. The subsequent down-regulation of Fas antigen expression in leukemic bone marrow cells is well correlated with the increased anti-apoptotic protein Bcl-xL expression. Thus, our results hinted that the activated pSTAT-3 and pSTAT-5 expressed by the leukemic bone marrow cells played vital roles in maintaining leukemic hematopoiesis and leukemic cell survivability in long term by up-regulating the anti-apoptotic protein Bcl-xL expression and blocking the Fas antigen-mediated apoptosis pathway in leukemia.

In conclusion, interactions of leukemic cells with bone marrow microenvironmental stromal cells and soluble mediators are fundamental for leukemic hematopoiesis, leukemic cell survival, disease progression and resistance to treatment. Stromal leukemic microenvironment could promote leukemic hematopoiesis by altering several adhesion receptor expressions and signaling pathways involving cell differentiation and death. Although the understanding of these processes is deepened, we need to characterize the relative contribution and importance of the

interactions and stromal cells involved in leukemogenesis. Exploring the behavior of leukemic stromal cells and malignant stromal components is a promising strategy for designing newly targeted therapies, ameliorating the disease progression, and limiting the resistance to conventional chemotherapy.

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