# Characteristics of Leukemia Stem Cells of Murine Myeloproliferative Disease Involving the Liver

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We studied surface phenotype of tumor cells and characterized leukemia stem cells in various cell populations with phenotypes of stem and committed precursor cells in the hierarchy of hemopoietic stem cells. Transplantable murine leukemia was used as the model. Bone marrow and liver cells from mice in the terminal phase of the disease were stained with antibodies to various surface markers and analyzed on a flow cytofuorometer. The cells were sorted by various differentiation markers using a system of magnetic separation Miltenyi Biotec® MACS® and then transplanted to syngeneic recipients. In some cases, limiting dilutions were used for measuring the concentration of leukemia-initiating cells. All transplanted cell populations caused death of recipients: c-kit$^-$CD45$^-$ over 23.9 days, c-kit$^+$ over 22.2 days, c-kit$^-$CD45$^+$ over 15.4 days, Ter119$^+$ over 18.2 days, and Ter119$^-$ over 17.7 days. The concentrations of leukemia cells determined by the method of limiting dilutions was 1 per 37,000 c-kit$^+$ bone marrow cells and 1 per 45 unsorted liver cells from sick animals. Thus, leukemia stem cells retain hierarchic organization in the studied model and can differentiate at least into myeloid and erythroid cells without loosing self-maintenance capacity. This model can be used for the study of regulation of self-maintenance mechanisms in various hierarchic populations of leukemia stem cells.

**Key Words:** *leukemia stem cells; transplantable leukemia; hierarchy of stem cells*

The existence of tumor stem cells was demonstrated by many researchers [6,7,10]. This is a subpopulation of tumor cells capable of initiating, producing, and maintaining the growth of tumors in xenogenic mouse models [11]. Serial transplantations of tumor stem cells lead to the development of neoplasia carrying the features of parental tumor, which reflects the differentiation potential of tumor stem cells. Hence, these cells are characterized by self-maintenance capacity and can produce differentiated progeny forming the bulk of the tumor [7]. In case of leukemia, a small population of cells capable of unrestricted self-renewal necessary for initiation and progress of the disease was also identified [3]. It should be noted that leukemia stem cells (LSC) can originate from the hemopoietic stem cells (HSC) [3,9] or from a more differentiated precursor acquiring self-maintenance potential due to a mutation [8]. Since both normal HSC and LSC are capable of self-maintenance, the difference between cancer stem cells and normal tissue stem cells is a critical issue if targeted therapies are to be developed. However, it remains unclear whether LSC are phenotypically and physiologically identical to normal HSC or they acquire the features of more differentiated precursors.

The aim of the present work was to study surface phenotype of tumor cells, including LSC, on the model of transplantable murine leukemia and to characterize LSC in different cell populations with phenotypes of stem and committed precursor cells in the hierarchy of HSC.

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## MATERIALS AND METHODS

Transplantable murine leukemia obtained in one mice after 3 courses of low-dose granulocytic CSF (25 μg/kg) [2] was used as the model. Intravenous injection of bone marrow (BM) or liver cells from a sick animal leads to leukemia development and death of syngeneic recipients within 13-55 days. In all animas, increased volume and weight of the liver (3.1±0.7 g *vs.* 1.4±0.1 g in healthy mice) and spleen (215±73 mg *vs.* 93±2 mg in healthy mice) were found.

For characterization of the surface phenotype of tumor cells and evaluation of changes in the BM and liver of recipient mice, the cells of BM and liver from sick and control animals were incubated with rat antibodies to CD45, CD11b (Mac-1 α-chain), Ly-6D (Mac-3), CD117 (c-kit), CD135 (Flt3) (BD Pharmingen) and then stained with secondary goat-anti-rat-FITC conjugated IgG/IgM (BD Pharmingen). Moreover, the cells were directly stained with FITC-conjugated antibodies to CD3 (hamster-anti-mouse-IgG1, FITC conjugated; BD Pharmingen). Before staining, the cells were incubated with 3% BSA to block nonspecific binding of antibodies. The level of nonspecific binding of antibodies was evaluated using isotypic control. To this end, the analyzed cells were incubated with total rat immunoglobulin fraction (all isotypes) isolated from rat peripheral blood. Cell viability was evaluated using 7-aminoactinomycin D (7AAD; Sigma).

The cells were analyzed on FACSCalibur flow cytometer (BD Biosciences); the data were processed using WinMDI v.2.8 and FlowJo v.7.2.4 (Tree Star Inc.).

For characterization of LSC of the tumor, BM cells from mice in the terminal phase of the disease were sorted using a magnetic separation system Miltenyi Biotec® MACS® according to manufacturer's protocol and then intravenouly injected to syngeneic recipients ($10^6$ cells per mouse). Each group consisted of 6-10 recipients. The following cell fractions were used in the study: c-kit$^-$CD45$^-$, c-kit$^+$ ($10^5$ cells per mouse), c-kit$^-$CD45$^+$, Ter119$^+$, Ter119$^-$, and unsorted BM and liver cells from sick animals in the terminal phase. The lifespan after injection for each animal and the presence of signs typical of this leukemia (enlarged and morphologically altered liver and enlarged spleen) in dead animals were taken into account.

For determining the concentration of leukemia-initiating cells, BM c-kit$^+$ cells and unsorted liver cells were injected in limiting dilutions (100,000, 20,000, 4000, 800, 160 cells per mouse and $10^6$, $10^5$, $10^4$, $10^3$, 100, 10, and 1 cells per mouse, respectively). Each group consisted of 10 recipient mice, except the group receiving 1 liver cell from sick animals (this group consisted of 20 recipients). The lifespan after injection of tumor cells for each animal and the presence of signs typical of the studied leukemia in dead animals were taken into account.

The concentration of leukemia-initiating cells was calculated using Poisson distribution [1].

The time between LSC divisions ($t_0$) was calculated from experimentally determined LSC concentrations in populations of BM c-kit$^+$ cells and unsorted liver cells from sick animals and from the lifespan of recipient animals in these groups.

The following assumptions were made during calculations:

1) the animal dies, when the tumor weight or the number of leukemic cells attains a certain threshold level C. This assumption is legitimate, because the involved liver weight in all dead mice of the experimental group was similar (3 g). Then C=const irrespective of the type of injected cells;
2) none LSC enter the resting phase of the cell cycle after division;
3) the division is symmetrical, *i.e.* one LSC gives rise to two equivalent LSC identical to the parent cells, which then also divide;
4) all LSC are equivalent;
5) the time between divisions is constant for all LSC.

First, we calculated $x$, the difference between the numbers of mitoses required for induction of animal death for c-kit$^+$ cells and unsorted liver cells. Let $a_0$ and $b_0$ be the concentrations of LSC in the population (per 1 mln. cells) and let $a$ and $b$ be the numbers of LSC among c-kit$^+$ cells of BM and unsorted liver cels, respectively.

Let $n_1$ and $n_2$ be the number of mitoses required for induction of death of the animal receiving $a$ or $b$ BM or liver LSC.

Then, $a\times2^{n1}=C$ **(1)**, $b\times2^{n2}=C$ **(2)**.

Since C=const, **(1)**=**(2)** and $a\times2^{n1}=b\times2^{n2}$, or $a\times2^{n1-n2}=b$ **(3)**.

Let $n_1$-$n_2$=$x$, then equation **(3)** can be presented as:

$$a\times2^x=b \quad \textbf{(4)},$$

where $x$ is the difference in the number of mitoses required for induction of animal death. If $10^5$ c-kit$^+$ cells of BM and $10^6$ unsorted liver cells were injected, $a_0\times10^5\times2^x=b_0\times10^6$, whence:

$$x=\frac{\lg(b_0/a_0)}{\lg 2}+3.3 \quad \textbf{(5)}.$$

Then, the time between divisions of LSC $t_0$ (in hours) is equal to the difference in the lifespan of animals receiving the test cells divided by the number of passed mitoses:

$$t_0=\frac{t_1-t_2}{\frac{\lg(b_0/a_0)}{\lg 2}+3.3} \quad \textbf{(6)},$$

where $t_1$ and $t_2$ are the lifespan of recipients in the group receiving c-kit$^+$ cells and unsorted liver cells, respectively, 3.3 is a correction for the difference in the number of injected cells ($10^5$ in the group receiving c-kit$^+$ and $10^6$ in the group receiving unsorted liver cells).

From the known time between LSC divisions, the lifespan after injection of each fraction, and the concentration of LSC among $10^6$ c-kit$^+$ cells, the number of LSC in each test population can be calculated ($C_{0i}$):

$$C_{0i} \approx a_0 \times 2^{\frac{t_1 - t_i}{t_0} - 3.3} \quad \textbf{(7)},$$

where $t_1$ is the time (hours) until animal death after injection of $10^5$ c-kit$^+$ cells, $t_i$ is the lifespan of recipients in $i$ population, and $a_0$ is the concentration of LSC in c-kit$^+$ cell population calculated using the method of limiting dilutions.

The data were processed statistically using Student $t$ test.

## RESULTS

For understanding of the etiology of this leukemia and evaluation of changes in BM and liver after injection of leukemic cells, surface markers of tumor cells should be identified. To this end, BM and liver cells from sick animals in the terminal phase of the disease were stained with antibodies to the following surface markers: CD45, CD11b (Mac-1 α-chain), Ly-6D (Mac-3), CD117 (c-kit), CD135 (Flt3), CD3 (Table. 1).

No significant differences by the above-listed markers were found between BM cells from sick and healthy mice. Nevertheless, the studied leukemia can be retransplanted by injecting $10^6$ cells from a sick donor to a syngeneic recipient. Hence, the presence of LSC in BM of sick animals capable of inducing leukemia in recipients does not significantly change the composition of hemopoietic cells in BM evaluated by the expression of surface markers at the moment of animal death.

No hemopoietic cells were found in the liver of healthy animals. The content of CD45$^+$ cells was 1.2±0.2%. The content of Mac-3$^+$ cells was 1.0±0.3%, *i.e.* all or almost all hemopoietic cells in the liver are Mac-3$^+$ cells. Mac-3 is expressed on the surface of tissue macrophages, which is confirmed by experimental data.

The liver from sick animals contained cells carrying all studied surface markers, though in small amounts. The difference by the content of CD45$^+$, c-kit$^+$, and Mac-1$^+$ cells between the sick and normal liver were significant ($p$<0.05). CD45$^+$ cells constituted 29.2±5.2% of all living cells in sick liver, which cardinally differ from the parameters of normal liver; c-kit$^+$ cells were also present among living liver cells from sick animals (5.1±1.2%). Apart from cells expressing early hemopoietic precursor marker c-kit, the liver from sick animals contained cells carrying surface marker Mac-1 (Table 1), which is expressed on more differentiated cells (granulocytes, macrophages, and dendritic cells).

Thus, the liver of sick animals is populated by hemopoietic tumor cells, including LSC, because injection of liver cells from a sick animal to a syngeneic recipient induces leukemia identical by its clinical picture to the initial disease.

Thus, cells carrying markers of early hemopoietic precursors (c-kit) and more differentiated cells (Mac-1) were detected in the liver of sick animals, and the next task was to characterize the surface phenotype of cells inducing the tumor (LSC). To this end, the following populations of cells isolated from BM of sick animals were injected to syngeneic recipients: c-kit$^-$CD45$^-$, c-kit$^+$, c-kit$^-$CD45$^+$, Ter119$^+$, Ter119$^-$ (Table 2). It was found that all these populations contained LSC. All recipients in all groups died (Fig. 1).

Using the method of limiting dilutions, we determined the concentration of LSC in the population of BM c-kit$^+$ cells (26 LSC per $10^6$ cells or 1 LSC per 37,000 cells) and among unsorted liver cells from sick mice (22,000 LSC per $10^6$ cells or 1 LSC per 45

**TABLE 1.** Percent of Living Cells Carrying Different Surface Markers in MB and Liver of Experimental and Control Animals

| Marker | BM from sick mice | BM from healthy mice | Liver from sick mice | Liver from healthy mice |
|---|---|---|---|---|
| CD45 | 79.6±2.6 | 83.6±1.3 | 29.2±5.2 | 1.2±0.2 |
| CD3 | 3.8±1.4 | 1.2±0.2 | 2.0±0.8 | 0.3±0.1 |
| c-kit | 1.5±0.8 | 3.4±2.2 | 5.1±1.2 | 0.1±0.1 |
| CD135 | 0.5±0.5 | 0.7±0.4 | 4.3±1.9 | 0.1±0.1 |
| Mac-3 | 1.2±1.0 | 13.4±5.1 | 6.1±1.5 | 1.0±0.3 |
| Mac-1 | 38.1±10.5 | 53.3±3.4 | 9.2±1.8 | 0.0±0.0 |

**TABLE 2.** Mean Lifespan of Recipients after Injection of Different Cell Populations

| Cell population | Mean lifespan of animals, days |
|---|---|
| Liver | 14.0 |
| c-kit$^-$CD45$^+$ | 15.4 |
| BM | 17.0 |
| Ter119$^-$ | 17.7 |
| Ter119$^+$ | 18.2 |
| c-kit$^+$ | 22.2 |
| c-kit$^-$CD45$^-$ | 23.9 |

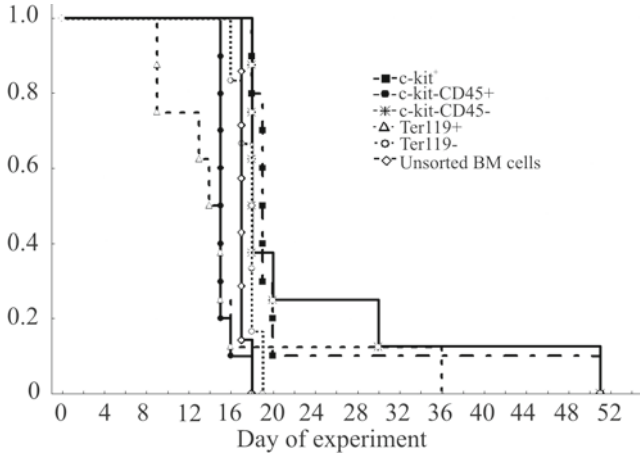


**Fig. 1.** Survival curves after injection of sorted cell populations from BM of sick mice. Here and on Fig. 2: Ordinate: proportion of survivors.

cells). The dynamics of animal death is presented in Figure 2.

Using formula **(6)**, we calculated the duration of mitosis in LSC (15 h). Then, using formula **(7)** we calculated the concentration of LSC in all analyzed fractions (Table 3).

In this model of leukemia, LSC were found in all analyzed BM cell populations (c-kit$^-$CD45$^-$, c-kit$^+$, c-kit$^-$CD45$^+$, Ter119$^+$, Ter119$^-$). Moreover, the time required for the disease onset decreased from c-kit$^-$CD45$^-$ to c-kit$^-$CD45$^+$ cell populations and attaining the minimum for cells populating the liver. The theoretical calculations allow determining the number of LSC in each population. These findings suggest that hierarchical structure of stem cells is preserved in this model, which agree with previous reports [8]. In contrast to previously descried models, in this model not single special cell type, but several cell populations exhibit the characteristics of LSC. Our findings suggest that the lower is the position of tumor progenitor cells in the hierarchy, the more rapidly the disease progresses to the terminal stage. It was demonstrated that in the hierarchy of normal HSC, the maximum index of proliferation was observed at the level of oligomonopotent precursors [4], which agrees with our findings. Moreover, promyelocytic leukemia, one of the most differentiated acute leukemia, is characterized by very rapid progress to the terminal stage without treatment [12]. It can be hypothesized that c-kit$^-$CD45$^-$ population contains earlier LSC than c-kit$^+$ population, because the time of leukemia development after injection of these cells is maximum. The HSC populations isolated without c-kit marker contains early precursors not carrying stem cell growth factor receptor and

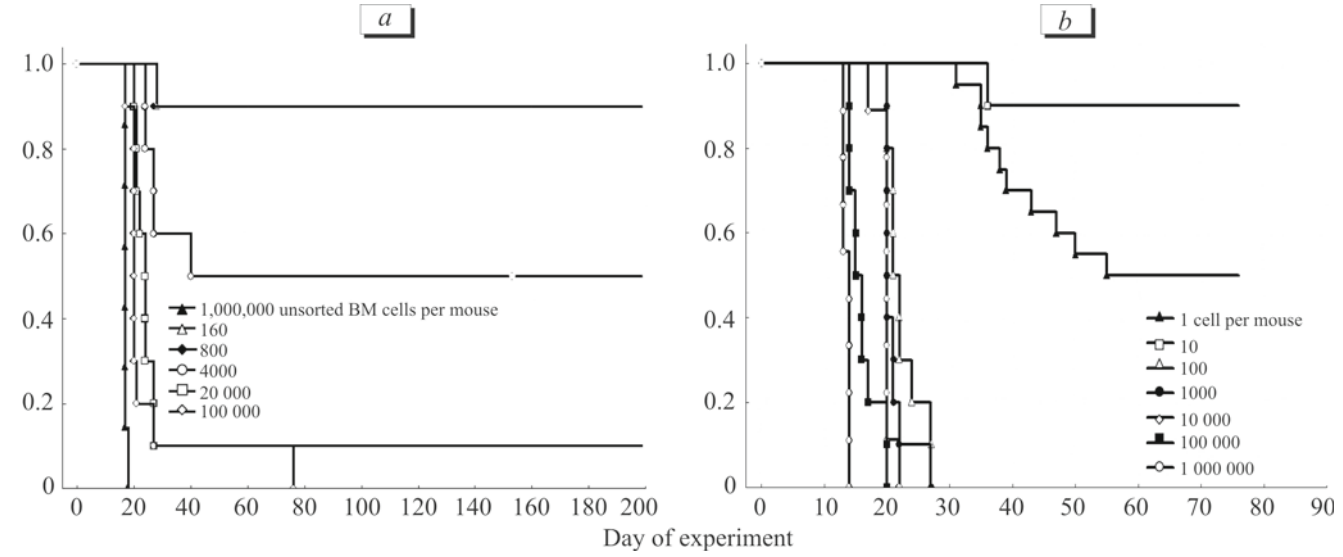


**Fig. 2.** Survival curves after injection of BM c-kit$^+$ cells (*a*) and unsorted liver cells (*b*) from BM of sick mice in limiting dilutions.

**TABLE 3.** LSC Concentration in the Analyzed Populations

| Cell population | LSC concentration (per 1 mln.) | 1 LSC per |
|---|---|---|
| Liver | 22 000.0 | 45 |
| c-kit$^-$CD45$^+$ | 4700.3 | 200 |
| BM | 804.9 | 1200 |
| Ter119$^-$ | 384.4 | 2600 |
| Ter119$^+$ | 222.3 | 4500 |
| c-kit$^+$ | 26.0 | 37,000 |
| c-kit$^-$CD45$^-$ | 0.4 | 2,500,000 |

capable of restoring hemopoiesis in lethally irradiated mice [5].

It can be concluded that in this model the characteristics of LSC are observed in cells carrying markers of early hemopoietic precursors and in committed myeloid and erythroid precursors. Thus, in the studied model leukemia stem cells retain hierarchic organization and can differentiate at least into myeloid and erythroid cells without loosing self-maintenance capacity. However, this did not significantly change the percent of these cells in BM. It was previously shown that the expression of apoptosis genes did not change in this disease [2]. It can be hypothesized that in this model of leukemia the number of differentiated precursor cells is strictly regulated by proliferation and apoptosis.

It can be concluded that the liver of sick animals is populated by hemopoietic tumor cells, including LSC. The obtained model allows us to study expression of genes responsible for self-maintenance and the mechanisms of regulation of this process in LSC carrying various differentiation markers. Moreover, short duration of mitosis comparable to division time for embryonic cells attracts our attention to genes regulating proliferation and passage through the cell cycle and to inhibitors of apoptosis.

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## REFERENCES

1. A. Banerjee, *Medical Statistics: Made Clear. An Introduction Course* [Russian translation] Moscow (2007).
2. A. E. Bigil'diev, N. V. Satz, A. L. Grishchuk, *et al*., *Byull. Eksp. Biol. Med*., **145**, No. 2, 234-238 (2008).
3. D. Bonnet and J. E. Dick, *Nat. Med.*, **3**, No. 7, 730-737 (1997).
4. D. Bryder, D. J. Rossi, and I. L.Weissman, *Am. J. Pathol*., **169**, No. 2, 338-346 (2006).
5. C. Z. Chen, L. Li, M. Li, and H. F. Lodish, *Immunity*, **19**, No. 4, 525-533 (2003).
6. J. E. Dick, *Nature*, **423**, 231-282 (2003).
7. O. Dreesen and A. H. Brivanlou, *Stem Cell Rev.*, **3**, No. 1, 7-17 (2007).
8. A. V. Krivtsov, D. Twomey, Z. Feng, *et al., Nature*, **442**, 818-822 (2006).
9. T. Lapidot, C. Sirard, J. Vormoor, *et al., Nature*, **367**, 645-648 (1994).
10. T. Reya, S. J. Morrison, M. F. Clarke, and I. L. Weissman, *Ibid*., **414**, 105-111 (2001).
11. J. C. Wang and J. E. Dick, *Trends Cell Biol*., **15**, No. 9, 494-501 (2005).
12. Z. Y. Wang and Z. Chen, *Blood*, **111**, No. 5, 2505-2515 (2008).