Study of leukemic stem cell population (CD34⁺/CD38⁻) and WT1 protein expression in human leukemic cell lines

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ABSTRACT

Background: Leukemic stem cells (LSCs) play a central role in relapse and refractory cases of leukemia patients. This cell has been found to resist to a conventional chemotherapy more than leukemic cells. Novel therapeutic strategy directly targets to eliminate the LSCs for eradication of the disease. Abnormal of leukemic cell proliferation is the main problem. The mechanism is involved in many proteins in cell signaling pathway. Wilms’ tumor 1 protein (WT1) is the transcription factor protein. It overexpresses and relates to leukemic cell proliferation but there is no report of WT1 protein expression in the LSCs.

Objectives: To compare the percent of LSC (CD34⁺/CD38⁻) population and WT1 protein expression levels in KG-1a, KG-1, and K562 cell lines.

Materials and methods: Leukemic cells were determined percent of LSC population and WT1 protein level by flow cytometry and Western blot analysis.

Results: The result showed that LSC population in KG-1a, KG-1, and K562 cells were 92.82±3.28, 75.95±4.83, and 0.44±0.51%, respectively. Almost cell population (over than 99%) in K562 cells was leukemic blast cells (CD34⁻). WT1 protein levels by mean fluorescent intensity (MFI) analysis in LSCs of KG-1a, KG-1, and K562 cells were 46.8±5.92, 59.54±4.65, and 183.42±17, respectively. By Western blot analysis, KG-1a cells showed the highest CD34 protein level while KG-1 and K562 cells were 35.68±11.01 and 3.56±3.56%, respectively, when compared to that of KG-1a (100%). Moreover, K562 cells showed the highest WT1 protein level (100%), followed by KG-1 and KG-1a cells with the expression values of 67.23±6.86 and 42.52±5.84%, respectively.

Conclusion: WT1 protein expression levels in LSCs of KG-1a cells was less than KG-1 and K562 cells. WT-1 expression is related with the leukemic cell proliferation rate.

Introduction

Cancer is a public health problem in the world which is approximately 13% of death cause. In 2012, there are more than 8.2 million of cancer patients and the World Health Organization (WHO) has predicted that there will be an additional 70% of cancer patients and will occur in developing countries in the next 20 years.¹ In Thailand, cancer is the first cause of death for several decades. In 2011, there were approximately 35,000 patients who died with cancers. Leukemia is a type of cancer that can be found throughout the world and in people of all ages, including Thailand. Leukemia is a well-known cancer which is 1 of the most top 10 common cancers. Currently, there are more leukemia patients which can be occurred in all ages. There were 2.65 million of patients who died with leukemia in 2012.¹ While there are estimated to die with leukemia about 2,500 patients in Thailand. Especially in
children, the incidence of leukemia was found in 53% of all cancers. Moreover, 74% of leukemia is a type of acute lymphoblastic leukemia (ALL). Nowadays, chemotherapy is the most popular for many cancer treatments, including leukemia. However, there are many leukemia patients who do not respond to chemotherapy because anti-cancer drugs cannot completely eliminate all of leukemic cells in patients. The remaining cells in the patients during treatment or after treatment is known as minimal residual disease (MRD). Leukemic stem cell (LSC) is always found in MRD. Previous reports showed that LSCs resisted to chemotherapeutic drugs more than normal leukemic cells after chemotherapeutic induction. They express CD34+ and CD38+ on their cell surfaces. Moreover, LSCs (CD34+/CD38+) could also self-renew and differentiate into leukemic blasts in the recipient mice. Cell surface proteins in LSCs are different from hematopoietic stem cells (HSCs) even if they are CD34+/CD38+. LSCs show different cell surface markers such as, CD44+, CD96+, and CD123+. High frequencies of CD34+/CD38+: LSC at diagnosis and after treatment help to predict relapse in AML.

In leukemic cells, Wilms' tumor 1 (WT1) protein is expressed as a biological marker and plays an important role in cell proliferation. It is overexpressed in leukemic cells when compared to normal blood cells. The decreasing of WT1 protein expression results in decreasing of leukemic cell proliferation. Thus, WT1 protein plays a key role in leukemogenesis. However, there is no report about WT1 in LSCs. We believed that all cell population mixtures of cell lines have LSCs, but the cell number population may be varied by the type of leukemic cell line. This study was to investigate percent population of LSCs in three leukemic cell lines and determine percent of WT1 expression in LSCs. KG-1, KG-1a, and K562 cells are leukemic cell lines to be used as the leukemic cell model in this study.

Materials and methods

Cell culture and condition

KG-1 (acute myeloblastic leukemia cell line), KG-1a (subline of KG-1 cell line), and K562 (chronic myelogenous leukemia cell line) were used as human leukemic cell line models in this study. K562 cells were cultured in RPMI-1640 medium (GIBCO™, Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (Capricorn, Ebsdorfergrund, Germany), 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin (GIBCO™, Thermo Fisher Scientific, MA, USA). KG-1a and KG-1 cells were cultured in IMDM medium (GIBCO™, Thermo Fisher Scientific, MA, USA). KG-1a and KG-1 cells were cultured in IMDM medium (GIBCO™, Thermo Fisher Scientific, MA, USA) supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin. All leukemic cell lines were cultured at 37 °C in a humidified incubator with 5% CO2.

Growth rate analysis of leukemic cell lines

KG-1a, KG-2, and K562 cells were seeded with complete medium in 24-well plate with the concentration of 1.0×10⁶ cells/well. Then, cells were counted with trypan blue dye exclusion method (0.4% trypan blue solution, Amresco®, OH, USA) for 6 days. Values for the measured property are plotted on a graph between total viable cell number and times.

Trypan blue dye exclusion method

After leukemic cells were harvested, cells were washed with ice-cold PBS, pH 7.4 for 3 times. Then, cells were resuspended with PBS, pH 7.4 and stained with 0.4% trypan blue solution at 1:2 dilution for counting cells on hemocytometer. The viable cell shows a clear cytoplasm because it can exclude trypan blue, while dead cells show a blue cytoplasm and vice versa in mechanism.

CD34+/CD38+ cell analysis by flow cytometry

KG-1a, KG-1, and K562 cells were harvested after cell concentrations reached 80% cell confluency. Then, cells were adjusted the concentration to 1.0×10⁶ cells/mL and blocked non-specific antibody binding with 10% AB serum in PBS for 30 min at 4 °C. After that, leukemic cell lines were incubated with FITC conjugated anti-CD34 and PE conjugated anti-CD38 monoclonal antibodies for 30 min at 4 °C. Cells were then washed with 1% BSA-PBS for 3 times and re-suspended with 1% paraformaldehyde. CD34+/CD38+ cells were analyzed by flow cytometer (FC500, Beckman coulter, CA, USA).

WT1 protein expression by flow cytometry

The leukemic cell lines were examined WT1 by flow cytometry. Briefly, leukemic cell lines (1.0×10⁶ cells) were blocked non-specific antibody binding with 10% human AB serum in PBS, pH 7.4 for 30 min at 4 °C. After that, cells were fixed with 4% paraformaldehyde for 20 min exactly. Then, cells were washed and permeabilized by permeabilization buffer (0.02% Na, 0.1% saponin, and 5% FBS in PBS, pH 7.4) and incubated with anti-WT1 antibody for 30 min at 4 °C. Cells were washed with permeabilization buffer and incubated with PE-Cy5.5 conjugated secondary antibody for 30 min at 4 °C. Finally, cells were washed and re-suspended with 1% paraformaldehyde for flow cytometry analysis.

Protein extraction and Western blotting

KG-1a, KG-1, and K562 cells were harvested after cells reached 80% cell confluency. Leukemic cells were harvested. The number of viable cells was determined using 0.4% trypan blue dye solution. Thereafter, total protein was extracted using RIPA buffer. Protein concentration was measured by the Folin-Lowry method. Protein was separated by 12% SDS-PAGE and then transferred to PVDF membranes. Membranes were blocked in 5% skim milk and probed by rabbit polyclonal anti-WT1 antibody (Santa Cruz Biotechnology, TX, USA), rabbit monoclonal anti-CD34 antibody (Santa Cruz Biotechnology, TX, USA) and rabbit polyclonal anti-GAPDH antibody (Santa Cruz Biotechnology, TX, USA) at dilution 1:1000. The reaction was followed by HRP-conjugated goat anti-rabbit IgG (Invitrogen™, CA, USA) at 1:20,000 dilution. The proteins were visualized using Lumina™ Forte Western HRP substrate (Millipore Corporation, MA, USA). Finally, the protein band signal was quantified by using a scan densitometer (Bio-Rad, CA, USA).
**Results**

**Cell growth of KG-1, KG-1a, and K562 leukemic cell lines**

Figure 1 shows the different patterns of leukemic cell growths. The growth rate of all leukemic cells showed lag phase in the first 3 days. After day 3, KG-1a and KG-1 cells showed low proliferation rate as compared to K562 cells.

**Statistical analysis**

Data are expressed as the mean±SD from triplicate samples of three independent experiments. The statistical differences between the means were determined using independent t-test. The differences were considered significant when the probability value obtained was found to be less than 0.05 ($p<0.05$) and 0.001 ($p<0.001$).

**Leukemic stem cell population in leukemic cell lines by flow cytometry**

In this study, KG-1a cells had the highest LSC (CD34+/CD38-) population with 92.82±3.28% by flow cytometry followed by KG-1 cells with 75.95±4.83%, while leukemic stem cell population in K562 cells were less than 0.5%. The K562 cell population mostly were blast cells (CD34-) which showed more than 99%. The result showed significantly different of LSC population among KG-1a cells and others two cell lines ($p<0.001$) (Table 1 and Figure 2).

**Table 1** Percentage of leukemic stem cell populations in cell lines.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>% Cell populations (Mean±SD)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CD34+/CD38</td>
</tr>
<tr>
<td>KG-1a</td>
<td>92.82±3.28</td>
</tr>
<tr>
<td>KG-1</td>
<td>75.95±4.83</td>
</tr>
<tr>
<td>K562</td>
<td>0.44±0.51</td>
</tr>
</tbody>
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*Data represents the mean±standard deviation (SD) of three independent experiments.*
To determine WT1 protein expressions in LSCs, all leukemic cell lines were determined and compared WT1 protein expression levels by flow cytometry. The result showed that K562 cells had the highest mean fluorescence intensity (MFI) of WT1 in leukemic cells with 183.42±17.44, followed by KG-1 and KG-1a with the MFIs of 59.54±4.65 and 46.8±5.92, respectively (Figure 3).

Figure 2. Leukemic stem cell populations in each cell line. (A) Flow cytometry analysis show cell populations in each quadrant region. Each cell line was stained with FITC conjugated anti-CD34 antibody and PE conjugated anti-CD38 antibody. (B) Data of LSC populations were represented as the mean value±SD of three independent experiments. Single asterisk (*) denotes a significant difference from each group at p-value<0.05 and double asterisk (**) denotes a significant difference in each group at p-value<0.001.

WT1 protein expression in leukemic cells by flow cytometry

To determine WT1 protein expressions in LSCs, all leukemic cell lines were determined and compared WT1 protein expression levels by flow cytometry. The result showed that K562 cells had the highest mean fluorescence intensity (MFI) of WT1 in leukemic cells with 183.42±17.44, followed by KG-1 and KG-1a with the MFIs of 59.54±4.65 and 46.8±5.92, respectively (Figure 3).

Figure 3. WT1 expression in leukemic cell lines. Each cell line was stained with FITC conjugated anti-CD34 antibody, PE conjugated anti-CD38 antibody, and anti-WT1 antibody with PE-Cy5.5 conjugated secondary antibody.
CD34 and WT1 protein expressions in leukemic cell lines by Western blotting

KG-1a cells showed the highest CD34 protein expression after determining by Western blotting (100%), while CD34 protein levels of KG-1 and K562 cells were significantly lower than KG-1a cells with the values of 35.68±11.01 and 3.56±3.56%, respectively (Figure 4). The WT1 protein levels showed that K562 cells had the highest WT1 protein level (100%), followed by KG-1 and KG-1a cells with 67.23±6.86 and 42.52±5.84%, respectively, compared to K562 cells (Figure 5). The results showed significantly different of WT1 protein expression between K562 cells and others two cell lines (p<0.05). Moreover, WT1 protein level of KG-1 cells was significantly higher (24.71%) than KG-1a cells (p<0.05).

Figure 4. CD34 protein expression levels in leukemic cell lines. (A) The levels of CD34 protein from KG-1a, KG-1, and K562 cell lines after incubation for 48 h were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer. Data are the mean values±SD of three independent experiments. Single asterisk (*) denotes a significant difference from each group at p-value<0.05 and double asterisk (**) denotes a significant difference in each group at p-value<0.001.

Figure 5. WT1 protein expression levels in leukemic cell lines. (A) The levels of WT1 protein from KG-1a, KG-1, and K562 cell lines after incubation for 48 h were assessed by Western blotting; GAPDH was used as the loading control. (B) The protein levels were analyzed with a scan densitometer. Data are the mean values±SD of three independent experiments. Single asterisk (*) denotes a significant difference from each group at p-value<0.05 and double asterisk (**) denotes a significant difference in each group at p-value<0.001.
Discussion

Nowadays, there are many reports which indicated both of acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) contain LSCs. LSCs are responsible for relapse and refractory cases to leukemia chemotherapy. Thus, remaining LSC in leukemia patient (minimal residual disease or MRD) is the main problem for leukemia treatment. LSCs are also very difficult to get rid of due to drug resistance and absence of drug response phenotypes. However, LSC population rarely presented in AML patient was found only 0.01-0.09%. This study is the first report to show the percent of LSCs in leukemia cell lines and relationship between LSCs and WT1 protein expression in leukemia cell lines.

In present study, three leukemic cell lines were used as model and investigated their growth rates and LSC populations for WT1 protein expression. LSC population in both KG-1a and KG-1 cells were more than 50% while LSC population in K562 cells were less than 0.5%. KG-1a cells had LSC population more than 90%. This result relates to the rate of cell growth in three cell lines.

WT1 protein have been reported as an oncoprotein which is involved in leukemic cell proliferation. Increase level of WT1 protein results in high rate of cell proliferation. This study, WT1 expression levels in KG-1a, KG-1, and K562 cells were different. WT1 expression level in K562 cells after flow cytometry and Western blotting showed the highest levels whereas, the lowest was found in KG-1a cells. This phenomenon relates to the growth rates of both cell lines. K562 cells demonstrated higher rate of cell proliferation than KG-1a cells from day 4 to 6. Furthermore, KG-1a cells showed the different LSC populations (92.82±3.28%) with 211-fold when compared to K562 cells (0.44±0.51%). LSC itself has lower activity when compared to leukemic blast cells. Almost cell population of K562 cell line is leukemic blast cells (CD34-). Thus, cell proliferation rate is higher than that of KG-1a cells (CD34+/CD38+). However, KG-1a cells showed the rate of cell proliferation and LSC population between KG-1a and K562 cells. KG-1a cell is less differentiated variant of KG-1 cell. Moreover, previous reports also indicated that LSCs expressed Bmi-1 gene which involved in the regulation of self-renewal and differentiation in LSCs. High level of Bmi-1 gene expression could lead to LSCs are mostly quiescent cell cycle which suggested that conventional anti-proliferative cytotoxic agents are minimally impacted against LSCs. In hematopoietic cell differentiation, HSCs (CD34+/CD38-), differentiate to be multipotent progenitor (CD34+/CD38+) and committed cells (CD34+/CD38-). HSC resides in the Lin-/CD34+/CD38+/CD90- fraction in bone marrow and CD34+/CD38- cells contained multiple progenitors while leukemia stem cells resided in an early CD34+/CD38+CD90- progenitor, while leukemic blasts were CD34+/CD38+ phenotype. Recently, there are previous reports demonstrated that the distinct surface protein expressions on LSCs as compared to normal HSCs, such as CD44, CD96e, and CD123. These antigens are useful for the isolation and identification of LSCs which required further study for investigation in KG-1a cells. Previous study showed that normal HSC-enriched population (Lin-CD34+/CD38-/CD90+) expressed CD96 weakly, while CD96+ AML cells are enriched for LSC activity. Thus, CD96 was reported as a LSC-specific marker in AML. However, WT1 protein have been reported as a biological marker in leukemia. This study reveals that WT1 protein expression also presents in LSCs even if lower than that of leukemic cells.

This is the first report to show the WT1 protein expression levels in LSCs. In present study, the result indicated that WT1 protein expression is inversely proportional to LSC population. Thus, KG-1a cells showed a low proliferative rate than KG-1 and K562 cells.

Conclusion

KG-1a cell represents LSC of leukemia patient. Furthermore, KG-1a cells also had WT1 expression which was a suitable target protein for further study. This study reveals the LSC population and WT1 protein levels in distinct phenotypes of 3 leukemic cell lines are different. These two factors (LSC population and WT1 protein) were found to relate to rate of cell proliferations. High level of WT1 protein indicates the high rate of cell proliferation as presented in K562 leukemic blast cells. On the other hand, low level was found in KG-1a leukemic stem cells. However, WT1 can be used as biological marker in LSCs. This research provides a basic knowledge for studying LSCs target therapy. In addition, there are some other proteins that also effect to proliferation and function of LSCs which should be investigated in further study.

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References


