Biologic Profile Evaluation of Mesenchymal Stem Cells in Co-culture with K562 Cells

Abstract

Background: Mesenchymal stem cells (MSCs) are among the most essential components of bone marrow (BM) microenvironment. Any infiltration of malignant cells or malignancy of BM cells could affect the fate of other cells in the BM microenvironment. Several studies have assessed the function and phenotype of BM-derived MSCs in leukemia patients, which have presented different results. Our goal in this research was to examine the cytogenetic and flow cytometric profiles as well as the growth of human umbilical cord MSCs (hUC-MSC) after co-culture with a chronic myeloid leukemia cell line, namely K562. Subjects and Methods: MSCs were isolated as a primary culture from hUC, co-cultured with K562 cells and examined in two groups of control (MSCs) and test (hUC-MSCs + K562 cells). Using karyotypic and flow cytometric techniques, cytogenetic and surface markers, as well as growth patterns of MSCs, were investigated in the two groups by plotting the growth curves. Results: MSCs cultured in the test group (together with K562 cells) were morphologically similar to those in the control medium. Cytogenetic analysis of MSCs in the test group indicated no chromosomal abnormalities; however, there were significant differences in the expressions of surface markers as well as in MSCs growth curves between control and test groups. Discussion/Conclusion: K562 cells do not have the ability to induce cytogenetic changes in MSCs, but they are capable of altering the expressions of surface markers as well as growth rates of MSCs.

Keywords: Bone marrow microenvironment, Co-culture, K562, Karyotype, mesenchymal stem cell

Introduction

Hematopoiesis involves a complex interaction between hematopoietic cells and bone marrow (BM) microenvironment, and several studies have demonstrated the association between abnormal BM microenvironment and pathogenesis of hematological disorders. Stromal cells are among the most important components of the BM niche, which are mainly differentiated from mesenchymal stem cells (MSCs) or fibroblasts. Morphological and functional changes in each component of BM microenvironment can reduce the differentiation capacity of MSCs, causing defective production of cytokines and growth factors as well as leading to immunophenotypic abnormalities. For example, recent studies show that extracellular vesicles (EVs) such as exosomes originating from MSCs or leukemia cells can positively (hemostasis) or negatively (malignancy) affect the BM microenvironment. and hence that exosomes derived from K562 cells can alter gene expression and cytokine secretion as well as suppressing BM-MSCs adhesion. On the other hand, MSCs can make changes in the BM microenvironment by secreting soluble factors like EVs as paracrine mechanisms. A study of MSC-EV function on leukemia cell growth showed that MSC-EV isolated from healthy donors inhibited tumors but that EVs from multiple myeloma (MM) patients led to an increase in tumor growth.

MSCs are the main constituent of BM microenvironment, contributing to the formation and function of BM microenvironment through supporting hematopoiesis and generating cells such as osteoblasts. MSCs are present in several tissues and are extensively used in clinical studies, given their unique advantages. In addition to their involvement in the BM niche, MSCs also participate in the formation of the tumor microenvironment, inducing growth and metastasis of tumor cells after homing at tumor sites. In fact, the interaction

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between MSCs and tumor cells leads to the secretion of signaling molecules that may be related to cell growth and apoptosis regulation in tumor cells. Recent evidence show that MSCs have a dual role in the suppression or promotion of tumor growth.\[^{[12,13]}\] For instance, research has shown that MSCs arrest tumor cells in the G1 phase of cell cycle and thus suppress tumor growth. On the other hand, MSCs reduce tumor cells’ apoptosis and can form a cancer SC niche where they support the proliferation of tumor cells.\[^{[14]}\]

Increasing pieces of evidence suggest that abnormalities in stromal cells residing in BM microenvironment can occur either physiologically or genetically. For instance, BM microenvironment defects may be related to the increase in age, leading to BM-failure syndromes such as MM, acute myeloid leukemia (AML), and myeloid dysplasia.\[^{[15,16]}\] BCR-ABL translocation has been reported in BM endothelial cells derived from chronic myeloid leukemia (CML) patients,\[^{[17]}\] and BM-MSC analysis of MM and MDS patients have indicated different gene expression profiles from those of healthy controls.\[^{[18]}\] Several clinical studies have demonstrated cytogenetic and functional abnormalities in BM-MSC of patients with leukemia [Table 1], but the question is whether these modifications in BM-MSCs could be induced by leukemic cells in vitro. This question has been put on trial in this project for the first time by investigating the biological profiles of human umbilical cord MSCs (hUC-MSC) after their co-culture with K562 cells. The hUC-MSCs and K562 cells have been used as a model of BM stroma and leukemia, respectively.

**Subjects and Methods**

The present study was experimental research.

**Isolation and culture of mesenchymal stem cells from human umbilical cord**

The isolation and culture methods are briefly explained elsewhere.\[^{[31]}\] The umbilical cord tissue explants were cultured in low-glucose Dulbecco’s Modified Eagle’s medium (DMEM) (Sigma-Aldrich, NC, US) supplemented with 10% fetal bovine serum (FBS) (Gibco, Gloucester, UK), 100 U/ml penicillin (Gibco, Erlangen, Germany), and 100 U/ml streptomycin (Gibco, Erlangen, Germany), subculture was performed when the cells reached 80%–90% confluence, and the cells of the third passage were used for experiments.

**Cell line**

Human CML cell line (K562) was cultured in RPMI-1640 medium (Sigma-Aldrich, NC, US), which was supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in a humidified incubator with 5% CO₂. The passage of K562 cells was done when they covered 80%-90% of the flask area.

**Co-culture of human umbilical cord-mesenchymal stem cells and K562 cells**

Direct co-culture was performed at 1:1 ratio, low-glucose DMEM/RPMI-1640 was used as the co-culture medium, and the co-cultured K562 cells were subsequently separated from hUC-MSCs by careful pipetting with ice-cold PBS.

**Cytogenetic analysis of human umbilical cord-mesenchymal stem cells**

MSCs from the third passage were co-cultured with K562 cells to explore whether K562 leukemia cells were able to change the cytogenetics of MSCs. The co-culture medium was changed every 3 days for 4 weeks. After this time, the karyotype analysis of MSCs was performed by the traditional Giemsa banding technique based on previously published reports.\[^{[32]}\]

**Immunophenotype analysis by flow cytometry**

In this research, flow cytometry was conducted for analyzing cell surface molecules of MSCs and comparing their expressions between test and control groups after 8 days to confirm the phenotypic profile of isolated hUC-MSCs. For this purpose, CD105-PE, CD73-PE, CD90-FITC, CD34-FITC, CD45-FITC, CD31-FITC, and HLA-DR-PE monoclonal antibodies (eBioscience, Thermo Fisher Scientific, San Diego, US) were used. The cells were incubated at 1 × 10⁶ cells/antibody at room temperature for 20 min in darkness. Afterward, the cells were rinsed with phosphate-buffered saline and incubated with their corresponding isotype control (eBioscience, Thermo Fisher Scientific, San Diego, US). The labeled cells were analyzed on a flow cytometer (Becton Dickinson) by collecting a minimum of 10,000 events.

**Growth curve analysis in human umbilical cord-mesenchymal stem cells**

MSCs were seeded into 6-well plates (SPL, Gyeonggi-do, South Korea) at a density of 1 × 10⁵ cells/well and maintained at 5% CO₂ within a humidified atmosphere for 24 h. Afterward, K562 cells were seeded at 1 × 10⁵ cells/well and co-cultured with MSCs in 6-well plates for 12 days. After 48 h, K562 cells were removed, and MSCs in well No. 1 from six wells of test and control group were trypsinized and counted. The number of cells in the two groups was subsequently compared. By day 12, this process was repeated until all six wells of both groups were counted. The growth curves of the two groups were compared over this period.

**Statistical analysis**

All statistical analyses were performed using SPSS Statistics V22.0 (IBM Company, NewYork, USA). The data were presented as mean ± SD and analyzed using the Mann–Whitney and repeated measures ANOVA between control and test groups. P < 0.05 was considered to indicate a statistically significant difference.
<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Method</th>
<th>Cytogenetic analyses</th>
<th>CD markers expressions</th>
<th>Other findings</th>
<th>References</th>
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<tbody>
<tr>
<td>AML</td>
<td>FISH</td>
<td>AML M4: (2;11), del (4), add (5) AML with MDS-related features: −Y, −4, der (5;17) −6, del (7), +8, +16, −18, −20, −22 AML M5: normal</td>
<td>➔ CD44, CD29, CD73, CD90, CD324 ($P&lt;0.05$)*</td>
<td>Reduced level of MCP-1 in BM stroma In hypoxic (5% oxygen) as compared to normoxic (21% oxygen) conditions, lower levels of GM-CSF, SCF, and TNF-α were detected</td>
<td>[18]</td>
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<td>NR</td>
<td>NR</td>
<td>➔ CD90, CD34, CD45 ($P&lt;0.05$)* ↓CD105 ($P&gt;0.05$)</td>
<td>The expression level of HSC quiescence regulating genes like ANGPT1 and SPP1 was significantly increased The proliferation capacity of AML-MSCs was variable, and they had normal or slow growth pattern MSCs demonstrated heterogeneous morphology, and cell viability was reduced in vitro</td>
<td>Structural abnormalities were detected in a significant proportion (48%) of MSCs Structural abnormalities have most often involved chromosomes 1, 7, and 10</td>
<td>[15]</td>
</tr>
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<td>Karyotype, FISH</td>
<td>AML M1: del (3), del (11), −22, del (8) AML M4: t (1;10), +13 AML M5b: t (2;13), −12 AML M7: t (7;9), t (7;10) RA: t (4;7), −16, −17, t (7;19), t (15;17) RAEB: der (7) t (1;7), del (17), t (1;3), del (2)</td>
<td>➔ CD105, CD90, CD29, CD33, CD34, CD14 ($P&lt;0.05$)*</td>
<td>No significant differences between MDS-MSC and normal MSC</td>
<td>[20]</td>
<td></td>
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<tr>
<td>MDS</td>
<td>Karyotype</td>
<td>−1, −20, −2, −10, −13, −22, −3, −12, −14, −16, −17, −15, −19, −5, −8, −11, −21</td>
<td>➔ CD105, CD29, CD34, CD14, CD68 ($P&lt;0.05$)*</td>
<td>In 5q- syndrome samples, overexpression of some genomic regions (7p22.3, 19p13.3, 19p13.11) were seen MSC layer morphology was different between normal controls and patients. Normal controls showed fibroblast-like shape but MDS-MSC seemed to be more thick and granular Impaired capacity of MDS-MSCs in differentiation to chondrocytic lineage was observed</td>
<td>[21]</td>
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<tr>
<th>Diagnosis</th>
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<th>Other findings</th>
<th>References</th>
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<tr>
<td>CML</td>
<td>Karyotype</td>
<td>Normal</td>
<td>CD11a, CD11b, CD14, CD29, CD31, CD34, CD44, CD45, CD105, GlyA, vWF, HLA-DR ($P&lt;0.05$)*</td>
<td>Normal cytokine expression pattern, CML-MSCs expressed SCF, G-CSF, and IL-6, but did not express TPO, IL-3, GM-CSF and M-CSF as normal MSCs did</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>FISH, nested PCR</td>
<td>Normal for t (9;22)</td>
<td>CD51/61, CD29, CD54, CD106, CD13, CD14, CD49e, CD49d, CD49b, CD34, CD45, HLA-DR ($P&lt;0.05$)*</td>
<td>CML-MSCs displayed normal hematopoietic supporting capacity</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>FISH, RT-PCR</td>
<td>A large proportion of BCR-ABL+ cells in CML stroma was seen, which these cells were CD14+ macrophages</td>
<td>CD14, CD45, CD34, CD105, HLA-DR ($P&lt;0.05$)*</td>
<td>Reduced levels of TNF, SCF and MIP-1α were observed in CML stromal supernatants</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>Karyotype, FISH</td>
<td>Normal for t (9;22)</td>
<td>CD14, CD45, CD49e, HLA class I ($P&lt;0.05$)*</td>
<td>CML stroma has reduced ability to support the growth of normal LTC-IC</td>
<td>[25]</td>
</tr>
<tr>
<td></td>
<td>Karyotype, FISH</td>
<td>CD73, CD90, CD105, CD45, CD34 ($P&lt;0.05$)*</td>
<td>Normal levels of TNF, SCF and MIP-1α were observed in CML stromal supernatants</td>
<td>CML-MSCs retained their ability to differentiate into osteoblasts</td>
<td>[26]</td>
</tr>
<tr>
<td>MPN ph−</td>
<td>Array-CGH</td>
<td>Loss 7pter-p22.2, loss 7p21.3, loss 7p21.3-p15.2, loss 7p12.3-p12.1, loss 7q11.22, gain 7q11.23-qter, Gain 7q22.1-qter, 5+ , 7+, Loss 11q13.2-q13.4, Loss 1q42.11-q44, loss 3p21.31-p11.1, loss 17q11.1-q11.2</td>
<td>CD73, CD90, CD105, CD45, CD34, CD13, CD33, CD14, CD80, CD31, HLA-DR, HLA class I ($P&lt;0.05$)*</td>
<td>The population doubling time of MPN MSCs was higher than normal MSCs</td>
<td>[27]</td>
</tr>
<tr>
<td>MM</td>
<td>Array-CGH, FISH, Real-time PCR</td>
<td>NR</td>
<td>Overexpression of IL-6</td>
<td>NR</td>
<td>[5]</td>
</tr>
<tr>
<td>ALL</td>
<td>Karyotype, FISH, Real-time RT-PCR</td>
<td>t (4;11) MLL-AF4</td>
<td>CD105, CD90, CD73, CD45, CD34, CD105, HLA-DR, CD19, CD14, CD166, CD106 ($P&lt;0.05$)*</td>
<td>V (D) JH monoclonal rearrangements were present in MLL-AF4+ leukemic blast cells, but were absent in BM-MSCs from infants with MLL-AF4+ B-ALL</td>
<td>[29]</td>
</tr>
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</table>
Results

Morphology of mesenchymal stem cells derived from umbilical cord

MSCs were effectively isolated from hUC tissues by adherent tissue culture method. After 1 week of incubation, fibroblast-like cells were detected around the umbilical cord tissue explants [Figure 1a], and a confluent monolayer was formed after 3 weeks [Figure 1b]. After co-culture, the morphology of MSCs from both groups was assessed, and the morphology of cells in the test group was found to be similar to that of the control group [Figure 2].

Cytogenetic analysis of human umbilical cord-mesenchymal stem cells

The cytogenetic karyotype of hUC-MSCs was analyzed in the two groups. No abnormal chromosomal changes (such as chromosome elimination, displacement, or chromosomal imbalance) were detected in the two groups [Figure 3].

Immunophenotypic analysis of human umbilical cord-mesenchymal stem cell

The immunophenotype of hUC-MSCs was assessed in test and control groups by staining for cell surface markers and flow cytometric analysis. The cells in the control group highly expressed typical MSC markers such as CD73, CD90, and CD105 but showed low expression levels of CD34, CD45, CD31, and HLA-DR. Nevertheless, the expressions of all markers (except for CD34 and CD45) significantly decreased on MSCs in the test group [Table 2].

Growth curve analysis

The proliferation of hUC-MSCs was not visibly different between the two groups during the first few days. On days 6–10, the proliferation rate accelerated, after which the growth decreased and plateaued [Figure 4].

Discussion/Conclusion

As mentioned, any change in BM microenvironment can affect BM components. Indeed, the importance of interaction between SCs and BM microenvironment has led to comprehensive investigations on BM microenvironment as well as concentrating on leukemia therapeutics because any damage to BM microenvironment may influence the function of SCs. MSCs have been identified as a major constituent of BM microenvironment affecting hematopoiesis, and since many studies have proven their role in increased tumor growth and metastasis, this research is focused on this group of BM niche cells.

The role of microenvironment in tumor development was first suggested by Paget in his “seed and soil” hypothesis. Recent reports have indicated that a number of genetic alterations in the stroma occur in the early stages of cancer. An unstable genetic stroma could facilitate the growth of malignant clonal cells. Evidence has shown that stromal cells play a crucial role in leukemogenesis, but the significance of cytogenetic abnormalities in stromal MSCs on the pathogenesis of leukemia has not been elucidated. Interactions between malignant hematologic cells and stromal microenvironment factors may direct stromal cells toward contribution to tumor progression. These interactions can also generate anti-apoptotic signals that, in turn, promote malignancy and resistance to treatment, thereby playing a critical role in patient survival and response to chemotherapy. In fact, structural and functional abnormalities of stromal cells might lead to disease progression or resistance to chemotherapy drugs.

Alternatively, BMT has been one of the treatment options for CML patients; however, the success of BMT has been limited due to GVHD and rejection of transplantation. Although the incidence of GVHD has been significantly decreased through the depletion of T-cells from BM or the application of purified HSC, such approaches have been associated with increasing transplant failure and recurrence of malignant...
disease. Therefore, the use of MSCs has been recently considered as an alternative approach because BM-MSCs is the only source capable of forming BM niche. Moreover, the hematopoietic environment of transplant recipients is destroyed by chemotherapy or radiation therapy, and MSCs can reconstruct the damaged stroma as an integral component of the BM niche. BMSCs can also reduce the chance of graft rejection through modulation and regulation of the immune system. Accordingly, the coexistence of HSCs and MSCs in transplantation increases transplantability but reduces GVHD and disease recurrence. For this reason, it is important to examine the functional and natural characteristics of MSCs to be aware of their normal or malignant nature.

In this research, we examined cytogenetics, cell surface markers, and growth patterns of normal hUC-MSC isolated from hUC following their co-culture with K562 leukemia cells. In previous investigations, MSCs derived from leukemia patients have been evaluated, but in this study, normal hUC-MSCs were studied for the first time. The reason behind the choice of this type of cell was to overcome certain ethical limitations and experimental interferences such as disease conditions and antitumor therapy, which may impose inappropriate constraints on our practical strategies.

In our study, no morphological changes were observed in hUC-MSCs following their co-culture with leukemia cells, which is in agreement with other reports investigating leukemia patients. Moreover, there was no difference in cytogenetic analysis results of MSCs compared to those of normal MSCs after long-term exposure to cancer cells. Our evidence of the cytogenetic profile of hUC-MSC was in line with other reports. Arnulf et al. in their research on BM-MSC derived from MM patients, stated that mesenchymal cells were normal in terms of cytogenetics, although they showed abnormal functional characteristics such as IL-6 overexpression [Table 1].

Our current explanations are consistent with previous findings on the cytogenetics of MSCs in CML patients. Greenberg et al., Wilson et al., Jootar et al., and Wohrer et al. showed that MSCs of CML patients lacked Philadelphia chromosome and had no common precursor with hematopoietic SCs (HSCs). Zhao et al. also reported a normal karyotype of MSCs isolated from CML patients and confirmed that their results did not change with an increasing number of passages. The similarity between cytogenetic disorders of MSCs and HSCs could be due to contamination of MSCs in culture with HSCs, which was for the first time revealed by Bhatia et al. who reported the presence of BCR-ABL+ cells among CML stromal cells and found that BCR-ABL+ cells included CD14+ BM macrophages as well as normal MSCs [Table 1]. However, in studies on CML patients, MSCs isolated from BCR-ABL-positive patients did not express t (9; 22) translocation, while MSCs of BCR-ABL-negative patients in initial passages showed JAK2V617F mutation in both MSCs and myeloproliferative neoplasms. Nonetheless,
other genetic abnormalities were detected in different chromosomes among 17% of BCR-ABL-negative patients.[44]

Confounding results have been reported in terms of myeloid disorders. Indeed, various studies have indicated that cytogenetic disorders are present in BM-MSCs of MDS, AML, and CML patients.[14] For instance, in the study of Zhang et al., there were chromosomal abnormalities in 57% of AML patients.[45] Cytogenetic analysis of AML and MDS patients in Blau et al. research indicated structural as well as numerical abnormalities of chromosomes[19][Table 1].

With respect to MSCs immunophenotype, decreasing CD90 expression has been reported in MDS-MSCs.[20] Studies by Campioni et al. and Carrara et al. have indicated significantly decreased expressions of CD104 and CD105 adhesion molecules. Other investigations showed insignificant differences in the expressions of some surface markers.[6,23] In this research, the expressions of CD90, CD105, CD73, CD31, and HLA-DR markers were significantly decreased compared to normal MSCs. Despite demonstrating chromosomal abnormalities in MDS-MSC, Flores-Figueroa et al. did not observe any significant difference in the functional profile of stroma (i.e., cell adhesion proteins, extracellular matrix proteins, ability to differentiate and support hematopoiesis in vitro) between patients and normals.[20] Huang et al. detected cytogenetic disorders of MSCs among AML patients that were different from leukemic blasts, but there were no changes in the expressions of cell surface markers and adhesion proteins (such as CD90, CD73, CD44, beta-integrin, and E-cadherin) between MSCs of patients in comparison with healthy controls[18][Table 1]. Since the expressions of CD90, CD73, and CD105 markers as well as the growth of MSCs were decreased after co-culture, reduced expressions of these markers may account for the reduction in growth pattern. As CD90 is a GPI-anchored protein and CD105 is an adhesion molecule, the reduction of the latter may have reduced cell adhesion to the bottom of the flask and delayed the growth of MSCs.

In this research, we observed a change in the growth curve of MSCs after co-culture with leukemic cells. Chandran et al. reported varying growth capacities in MSCs isolated from AML patients and hence that the isolated cells of some patients had normal growth patterns, but others showed...
decreasing growth patterns. However, contrary to our experiment, Zhao et al. showed the normal growth curve of MSCs taken from normal CML patients. Moreover, studies on MSCs isolated from MM patients have reported normal growth patterns of these cells. Further studies are needed to discover whether chromosomal and phenotypic disorders lead to functional changes in the stroma and how these alterations affect disease progression and outcome of leukemia patients.

Conclusion

In this study, cytogenetic analysis of MSCs showed that leukemic cells were not able to cause genetic changes in MSCs following 1-month exposure to normal hUC-MSCs. Nevertheless, the expressions of most surface antigens in MSCs were significantly reduced after co-culture with leukemic cells compared to normal MSCs. The growth patterns of MSCs showed a significant decrease after co-culture with leukemia cells. Our findings suggest that significant functional changes of MSCs in patients with leukemia are a basis for future studies, which help predict the response to the treatment or prognosis of the disease.

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Conflicts of interest

There are no conflicts of interest.

References


