Mesenchymal stem cells promote leukaemic cells aberrant phenotype from B-cell acute lymphoblastic leukaemia

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BACKGROUND AND OBJECTIVES: The role of bone marrow-mesenchymal stem cells (BM-MSC) in leukaemic cell control is controversial. The purpose of this work was to evaluate BM-MSC role regarding the viability, proliferation and immunophenotype of normal B-cell precursors from control (Ct) patients and leukaemic cells from B-acute lymphoblastic leukaemia (B-ALL) patients.

PATIENTS AND METHODS: BM-MSC were isolated and characterised from voluntary donors. Mononuclear cells isolated from Ct and B-ALL bone marrow samples were cultured in the presence or absence of BM-MSC for 7 days. Cell viability was determined with LIVE/DEAD and proliferation index evaluated by CFSE labelling. Cell population immunophenotypes were characterised by estimating CD19, CD10, CD20 and CD45 antigens by flow cytometry.

RESULTS: After co-culture, B-ALL cells exhibited higher viability (20–40%) as compared to just cells (3–10%). Ct and B-ALL absolute cell counts were higher in the presence of BM-MSC (Ct: 25/mm³ cf 8/mm³, B-ALL: 15/mm³ cf 3/mm³). Normal B-cell subpopulations in co-culture had increased expression of CD19 and CD10 (Pre-B) and CD45 and CD20 antigens (Pre-B). B-ALL cells co-cultured with BM-MSC showed an increase in CD19 and CD20, although the greatest increase was observed in the CD10 antigen.

CONCLUSIONS: Lymphoid cell maintenance, at early stages of differentiation, was significantly promoted by BM-MSC in normal and leukaemic cells. Co-cultures also modulated the expression of antigens associated with the B-ALL asynchronous phenotype as CD10 co-expressed with CD19 and CD20. To our knowledge, this is the first time that CD10, CD19 and CD20 leukaemic antigens have been reported as being regulated by BM-MSC.

Hematopoiesis is a physiological process which depends on hematopoietic stem cell (HSC) ability to self-renew, proliferate or differentiate.1,2 Since Schofield’s niche was proposed 30 years ago, researchers have learned that more than 10 cell populations may interact with HSC through adhesion molecules and/or different soluble factors.3 Bone marrow mesenchymal stem cells (BM-MSC) are particularly important hematopoietic regulators due to their capacity to differentiate into specialised stromal cells and produce soluble factors promoting primitive hematopoietic cell maintenance.4–6 This capacity has been extensively used for in vitro HSC expansion, preserving stemness properties, that is, self-renewal and differentiation potential.7,8 This has been useful in preserving myeloid populations but it has not been fully investigated in lymphoid progenitors.1 Also, few studies dealt with human lymphocyte
immunophenotypic changes in vitro co-cultured with BM-MSC. In spite of this, the differentiation pattern of committed progenitors such as the B-cell lineage has been accurately dilucidated.

B-cell lineage hierarchical organisation begins with Pre-pre B-cells, where CD19 is first expressed together with CD10, CD34, CD79a and nuclear terminal deoxynucleotidyl transferase (TdT) markers. These cells then differentiate into Pre-B cells expressing cytoplasmic Ig 

\[ \text{Ig} \]

\[ \text{cy} \]

and drug resistance, however, their role in B-ALL immunophenotype regulation has only been established in BLIN-2 cell lines, unfortunately without complete characterisation of stromal cells.

The present study has evaluated the effect of BM-MSC on cell viability, proliferation and the immunophenotype of primary leukaemic cells isolated from B-ALL patients compared to cells isolated from patients not suffering from haematological neoplasia. This could be useful since most studies have been performed with in vitro established cell lines and may not reflect the real situation in vivo.

MATERIALS AND METHODS

Isolation and characterisation of BM-MSC

BM aspirates were obtained from the iliac crest of volunteer donors after signing the informed consent form given by the ethical committee of Hospital Universitario San Ignacio. BM-MSC were isolated and cultured as previously published and they were used in passages 3–5 for co-culture assays. BM-MSC phenotype assessed by flow cytometry with following monoclonal antibodies (MAbs): FITC mouse anti-human CD73 (clone AD2, BD Pharmingen™), PE mouse anti-human CD45 (clone 2D1, BD Biosciences) and APC mouse anti-human CD34 (clone 581, BD Pharmingen™). Data was acquired in a FACS Calibur cytometer (BD Biosciences, San Jose, CA, USA). For data analysis PAINT-A-GATE software (BDB) was used. BM-MSC functional assay were performed according to previous reports with StemPro Osteogenesis, Adipogenesis and Chondrogenesis Differentiation Kits (Invitrogen).

Immunophenotype evaluation of controls and patients with B-LLA

Patients with B-ALL (n = 6) were initially diagnosed at the Hospital Universitario San Ignacio’s Flow Cytometry Service in Bogotá (Colombia, South America). B-ALL diagnosis was established according to the WHO criteria. The B-ALL immunophenotype in all cases was CD19+, CD34+, TdT+ CD10+ with CD20 asynchronous expression in four of the six samples. Percentage of infiltrated leukaemic blasts was elevated with an average of 84.6% (interval: 73.2–92.1%). The mean percentage of normal BM cells in all samples was 15%.

After morphological and immunophenotypic diagnosis, BM samples were sent to our laboratory, where
Mononuclear cells (MNC) were isolated by Ficoll density gradient centrifugation (Histopaque $d = 1.077 \text{ g/cm}^3$, Sigma–Aldrich, USA) and frozen at $-70^\circ\text{C}$ (Ct and B-ALL samples were frozen for a maximum period of 30 days). After cell thawing, immunophenotype was repeated (here on, basal immunophenotype) with the following MAbs panel: FITC mouse anti-human CD19 (clone HIB19, BD Pharmingen), PE mouse anti-human CD10 (clone HI10a, BD Biosciences), PerCP mouse anti-human CD45 (clone 2D1, BD Biosciences) and APC mouse anti-human CD20 (clone L27, BD Biosciences).

Control patients (Ct) ($n = 6$) BM samples were obtained from volunteer donors with confirmed non-malignant blood disorders and evaluated in the same way. For flow cytometry analysis, BM-MNC from Ct and B-ALL patients were selected using the CD19/side scatter (SSC) dot-plot and CD10/side scatter (SSC) dot-plot, respectively. Data was acquired in a FACS Aria-II flow cytometer.

Figure 1. Morphology of BM-MSC. BM-MSC displayed a fibroblast-like morphology after 8 (A) and 15 (B) days in culture (Olympus CK2, magnification 10x. Scale bar: 100 μm). Photographs were taken with a Sony Cybershot DSC-W7 digital camera, and colours were corrected with Adobe Photoshop.

Figure 2. BM-MSC immunophenotype. Histograms showing antigen expression in freshly BM-MSC: From left to right CD105, CD73 (mesenchymal antigens) and CD45 and CD34 (hematopoietic antigens); BM Sample 01 (A–D) and 02 (E–H). Dashed line: antigen expression; solid line: auto-fluorescence control.
(BD Biosciences, San Jose, CA, USA) and analysed in multiparameter PAINT-A-GATE software (BD Bioscience).

**Cell morphology evaluation and culture conditions**

Fresh BM-MNC samples from Ct and B-ALL were cytospun and stained with Wright's dye. Photographs were taken with a Sony Cybershot DSC-W7 digital camera and the colours corrected with Adobe Photoshop software.

After cell thawing, BM-MNC (Ct or B-ALL) were cultured in two experimental conditions: (i) $2 \times 10^4$ cells were plated in direct contact with confluent BM-MSC feeder cells in RPMI medium; and (ii) $2 \times 10^4$ cells were plated without BM-MSC. Cultures were maintained in a humidified atmosphere at 37 °C for 7 days.

**Viability and absolute count of Ct and B-ALL cells**

Ct and B-ALL BM-MNC viability were evaluated before and after culture using LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (405 nm excitation – Molecular Probes). Cells were counted by flow cytometry using CountBright™ Absolute Counting Beads (Molecular Probes). Absolute cell numbers per sample volume were calculated using the following formula: 

$$\frac{\text{number of cell events}}{\text{number of beads events}} \times \left(\frac{\text{beads/50 \mu l}}{\text{volume of sample}}\right)$$

![Figure 3. BM-MSC multipotency assay. Cultured BM-MSC were induced to differentiate into adipocytes (A and B), chondrocytes (C and D) and osteoblasts (E and F) in defined media for 2–3 weeks. Differentiation was evidenced by lipid vacuole detection by Sudan black staining (10x) (A and B), positivity for glycosaminoglycans with safranin “O” staining (5x) (C and D) and calcium deposits were detected by Von Kossa staining (5x) (E and F) (Olympus CK2, Scale bar: 100 \mu m).](image-url)
Cell division estimated by carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling
Proliferation assays for CFSE stained BM-MNC from Ct and B-ALL cells were performed as previously described. A fraction of BM-MNC was serum deprived for 48 h to establish the highest CFSE mean fluorescence intensity (cell with no proliferation). Both CFSE+ cells were cultured as described above. Decrease in mean fluorescence intensity (MFI) of CFSE was determined using the cytometer FACS-II Aria (BD Biosciences). Data analysis was performed with the application "proliferation analysis" available in the FlowJo 7.6.5 program (TreeStar, Inc) to calculate the proliferation index (PI).

Evaluation of Ct and B-ALL cells immunophenotype after co-cultivation
Evaluation of cells immunophenotype was carryout after 7 days of culture employing the same antibodies and software used for the basal phenotype estimation. Reliable discrimination between BM-MSC and Ct or B-ALL cells was possible according to (i) their different forward-scatter (FSC) and side-scatter (SSC) signals, and (ii) cells discrimination for antigen

Figure 4. Cell morphology from one Ct and two B-ALL BM samples. Microscopic evaluation of three BM samples, as examples: (A) Ct, (B) B-ALL (Pre-pre B), (C) B-ALL (Pre-B) (scale bar: 30 μm) (Olympus CK2, magnification 100x).

Figure 5. Cell viability from Ct and B-ALL patient samples. Percentage of viable cells assessed by flow cytometry in (A) Ct and (B) B-ALL patients in basal conditions (before culture) compared to cells cultured for 7 days in the absence (−) or presence (+) of BM-MSC.
Hierarchical clustering analysis
The hierarchical clustering analysis was carried out with the MFI information for each marker and the ratio between the MFI values for each marker in B-ALL neoplastic cells and the MFI value for the same marker in Ct. Clustering was performed by median centring and normalisation of the fluorescence ratios. A logarithmic (base 2) transformation was applied to the ratio values in individual data sets. The resulting normalised log 2 ratios were used for the Cluster and Tree View programs according to procedures previously described.

Statistical analysis
The distribution of the data was analysed using the Shapiro-Wilk test. Statistical tests were used for non-parametric data. To compare culture conditions (Ct or B-ALL) the Wilcoxon signed-rank test was used using Statistix 6.0 and GraphPad Prism 5.0 for graphics. Results were considered significant when p value was below 0.05.

RESULTS
BM-MSC characterisation
Following International Society of Cellular Therapy recommendations, BM-MSC were characterised by morphology (Figure 1A and B) and immunophenotype (Figure 2). All experiments were performed with BM-MSC isolated from two independent donors (M01 and M02). BM-MSC were negative for CD34 and CD45 and more than 98% were positive for CD73 and CD105 antigens (Figure 2). Differentiation assays showed cells with lipid vacuoles (adipogenic lineage), glycosaminoglycans (chondrocytes) and alkaline phosphatase activity (osteoblasts) (Figure 3).

Figure 6. Absolute cell counts from Ct and B-ALL BM samples. Comparative analysis in absolute numbers of (A) Ct and (B) B-ALL cells after 7 days culture without (−) or with BM-MSC (+). The mean value is indicated by the horizontal line.

Figure 7. Ct and B-ALL BM samples proliferative index. Significant differences in the PI of (A) Ct and (B) B-ALL cells were observed after 7 days culture in co-culture as compared to cells lacking BM-MSC *(p < 0.05).
Figure 8. Ct and B-ALL cell basal immunophenotype. FSC and SSC bivariate dot plot beads (red dots in R1 region) and cells (R2 region) were gated from representative samples (A and F). Antigens expression is indicated (B–E) for Ct (C42 sample) and (G–J) for B-ALL (L15 sample). Panel E shows normal B-cell lineage in Ct: (1) Pre-pre-B, (2) Pre-B and (3) B-cells.
Morphological characterisation of Ct and B-ALL cells

Fresh BM-MNC cell samples from Ct included myelocytes, promonocytes, granulocytes and erythroid precursors (Figure 4A). In contrast, BM-MNC samples from B-ALL were highly infiltrated (see Materials and methods) with blasts cells having prominent nucleoli and basophilic-agranular cytoplasm (Figure 4B and C; two patients’ samples).

Cell viability, absolute counts and proliferation index before and after co-cultures

Cell cultures were done with and without BM-MSC. After 7 days of culture viability, percentage in Ct and B-ALL cells without BM-MSC had significantly decreased as compared to basal cell viability (before culture) \( (p < 0.05) \) (Figure 5A and B).

Ct and B-ALL cells absolute counts were estimated after 7 days of culture with and without BM-MSC. More cells were observed in Ct samples when co-cultured (Figure 6A). Similar results were observed in B-ALL cells (Figure 6B). In both Ct and B-ALL samples, the proliferation index (PI) was higher when co-cultured with BM-MSC (Figure 7A and B) \( (p < 0.05) \).

Immunophenotypic analysis of Ct and B-ALL patient cells

Basal immunophenotype was analysed in all samples. Identification of B-cell precursors from Ct (Figure 8A) was based on CD45 expression in CD19+ cells co-cultured with BM-MSC showed higher viability compared to cultures without BM-MSC \( (p < 0.05) \) (Figure 5A and B).
B-ALL leukaemic cells show homogeneous FSC and low SSC signals (Figure 8F), CD19 (Figure 8G), bright CD10 (Figure 8I), asynchronous CD20 (Figure 8J) with low CD45 expression (Figure 8G and H), consistent with a leukaemic immunophenotype. After 7 days of culture, three differentiation stages could be distinguished in Ct B-lineage cells immunophenotype: Pre-pre-B, Pre-B and B-cells (Figure 8E). Therefore, the immunophenotype analysis was made over these subpopulations. In Ct cells co-cultured with BM-MSC, CD19 and CD10 expressions had increased in Pre-B subpopulation (Figure 9A–G). CD45 and CD20 expression had increased in Pre-B subpopulation (Figure 9E–K), while only CD19 expression decreased in B-cells (Figure 9C).

Concerning B-ALL cell immunophenotype analysis, CD19 expression had increased following BM-MSC co-culture, compared to cells without BM-MSC (Figure 10A). CD45 expression did not change after the co-culture (Figure 10B). The greatest increase was in CD10 (Figure 10C) and CD20 (Figure 10D) antigens.

For a global assessment of immunophenotype patterns between Ct and B-ALL cells a hierarchical clustering analysis was done after culture. Figure 11 shows the clusters at different experimental conditions, confirming CD19, CD10 and CD20 overexpression in B-ALL cells after co-culture with BM-MSC. Hierarchical clustering analysis revealed an association between Pre-pre B-cells and B-ALL in basal conditions (Figure 11A) and, after co-culture, with BM-MSC (Figure 11C). Ct and B-ALL analysis revealed no association between cell groups after culturing without BM-MSC (Figure 11B).

DISCUSSION

Several authors have demonstrated leukaemic cell viability maintenance by BM-MSC. Zhang et al. demonstrated that LSC isolated from CML patients can produce cytokines such as G-CSF which alter stromal cell CXCL12 secretion and HSC normal growth but favouring leukaemic cell proliferation, this being a bidirectional interaction between LSC and stromal cells.

Our results regarding BM-MSC influence of leukaemic cell viability maintenance agreed with these reports. Herein, we have demonstrated that BM-MSC cells from volunteer donors promoted cell survival of normal as well as leukaemic cells (B-ALL), even after thawing and culturing for 7 days (Figure 5). This showed that BM-MSC had a pro-viability effect on this type of leukaemic cell. We also found that BM-MSC did not inhibit Ct and B-ALL cell proliferation;

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Figure 10. B-ALL cells immunophenotype analysis. Antigen expression in basal and in the different culture conditions (7 days of culture without (−) and with (+) BM-MSC): (A) CD19, (B) CD45, (C) CD10 and (D) CD20. p Values are indicated.
conversely, the absolute counts (Figure 6) and PI (Figure 7) increased after co-cultivation. Notably, our results with BM-MSC isolated from two different individuals (Figure 2) were very similar (data not shown), indicating that the source of bone marrow cells did not affect the outcome.

Recent reports have proved that mesenchymal cells isolated from the umbilical cord can inhibit B-lymphocyte differentiation. However, herein we have shown that it changes according to lymphoid cell differentiation stage (Figure 9A–C), because CD19 expression only decreased in B-cells (Figure 9C). It is known that CD19 antigen is involved in B-cell lineage development, differentiation, activation and survival. It can thus be assumed that BM-MSC promote early lymphoid progenitor viability (Pre-pre B) by increasing CD19 expression as part of lymphopoiesis.

Figure 11. Hierarchical cluster analysis of markers differentially expressed in B-ALL cases compared to Ct populations. (A) Basal, (B) without BM-MSC and (C) co-culture (dotted line indicates the association between Pre-pre B and B-ALL cells). The columns show normalised log2 ratios for MFI values calculated for each analysed marker. Protein expression levels are represented by colour codes. Red indicates over-expression respecting the mean; and green represents under-expression compared to the mean value. Colour intensity denotes deviation magnitude (scale extends from ratio −3.00 to +3.00 log2 units).
homeostasis, whilst BM-MSC has an immunomodulating effect in more differentiated cells (B-cells) by decreasing CD19 antigen expression.

The immunophenotype changes of B-ALL cells induced by BM-MSC were more dramatic. After 7 days, B-ALL cells cultured with BM-MSC increased CD19, CD10 and CD20 compared to baseline and without co-culture with BM-MSC. CD10 antigen is a member of the membrane-bound zinc-dependent endopeptidases, a protein family regulating different cellular processes by peptide-substrate cleavage which can inhibit cellular differentiation. Also, CD10 has been used as a cell surface marker for stem cell population identification in breast cancer and for sphere-forming cells, suggesting that this protein can identify stem/progenitor populations. This finding is important since BM-MSC increased CD10 expression in cell populations during early stages of differentiation (Pre-pre-B and B-ALL cells), which might indicate that BM-MSC has a role in regulating more primitive hematopoietic progenitors and this, in turn, could be involved in leukemogenesis.

BM-MSC also increased CD20 expression in B-ALL. This antigen is a calcium-permeable cation channel known to accelerate the Go/G1 progression induced by insulin-like growth factor-I (IGF-1). Interestingly, BM-MSC produce IGF-1 inducing leukemic cells to increase CD20 expression, as observed here. Interestingly, CD20 expression in B-ALL is asynchronous, so it would have been expected that BM-MSC could also favour an asynchronous phenotype.

Previous studies have shown that the bone marrow microenvironment provides higher viability and resistance to chemotherapy for leukaemic cells, thereby increasing the likelihood of relapse in patients with B-ALL. It has been observed that increased CD20 expression is associated with a shorter time to remission and disease survival. The efficiency of monoclonal antibody therapy, such as rituximab (anti-CD20), has shown that this antigen is important in B-ALL pathophysiology. The same could be considered concerning the CD19 antigen, since the use of blinatumomab (anti-CD19) has also led to promising results. Moreover, increased CD10 expression has considered a good prognosis factor in children having B-ALL, although others have considered that its prognostic value should not be considered independently and that it should be analysed within the context of the other antigens. This study has clearly demonstrated that BM-MSC increased CD10, CD20 and CD19 expression in blasts isolated from patients having B-ALL. Since blast infiltration was high in our patients, we believe that haematogones and the contribution of other primitive normal cells to the immunophenotype must have been very low, or non-existent. It is likely that this BM-MSC-induced antigen over-expression is a blast protection-inducing mechanism, probably responsible for increased relapse.

CONFLICT OF INTEREST
None declared.

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