ORIGINAL ARTICLE

Mesenchymal Stem Cells Inhibit Proliferation of Lymphoid Origin Haematopoietic Tumour Cells by Inducing Cell Cycle Arrest

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SUMMARY

We have previously shown that mesenchymal stem cells (MSC) inhibit tumour cell proliferation, thus promising a novel therapy for treating cancers. In this study, MSC were generated from human bone marrow samples and characterised based on standard immunophenotyping. When MSC were co-cultured with BV173 and Jurkat tumour cells, the proliferation of tumour cells were profoundly inhibited in a dose dependent manner mainly via cell to cell contact interaction. Further cell cycle analysis reveals that MSC arrest tumour cell proliferation in G_0/G_1 phase of cell cycle thus preventing the entry of tumour cells into S phase of cell cycle.

KEY WORDS:
Mesenchymal Stem Cells, Tumour Cells, Proliferation, Apoptosis, Cell Cycle Arrest

INTRODUCTION

A substantial amount of research over the past two decades has resulted in greater understanding of the human adult stem cell biology not only in the basic sciences but also their implication in therapeutic usage. Following the initial enthusiasm, it is now becoming clear that the use of adult stem cell transplantation is an important tool in the treatment of various malignancies, tissue repair and tissue regeneration[1,2]. Adult stem cells are multipotent stem cells, which can be isolated from postnatal tissues, most commonly from bone marrow. Examples of adult stem cells are haematopoietic stem cells (HSC), mesenchymal stem cells (MSC) and other types such as neural, epithelial, oval and satellite cells[3,4].

Mesenchymal stem cells (MSC) constitute a rare non-haematopoietic population in the adult bone marrow (BM) which can be defined according to its ability to self-renew and differentiate into tissues of mesodermal origin (osteocytes, adipocytes, chondrocytes)[5]. In line with this, MSC serve as progenitors for bone marrow stroma and thus play a crucial role in supporting haematopoiesis by providing haematopoietic progenitors the necessary cytokines and cell contact mediated signals to self-renew and/or differentiate[2]. Lately, MSC were utilised to control the unwanted immune responses due to their immunosuppressive activity[6]. The immunosuppressive activity exerted by MSC on immune cells such as T, B and dendritic cells is mainly due to cell cycle arrest and not specific to cell types[7,8]. Therefore, the implication of cell cycle arrest of MSC on cell cycle dysregulated tumour cell proliferation may provide an alternative approach to cancer treatment.

This would be an ideal approach since traditional methods of targeting the tumour cells by non-specific radiotherapy and chemotherapy have not yield satisfactory outcomes as seen in many cancer remission cases. This conundrum leads exploration of new concepts in cancer pathogenesis and its treatment whereby introduction of cellular therapy and targeting the origin of cancer stem cells have been actively investigated. In line with this our previous work has shown that mesenchymal stem cells (MSC) serve as potential agent that limits growth of various tumour cells[9]. Although the anti-proliferative effect of MSC has been clearly demonstrated but the molecular interaction and signalling pathways between MSC and tumour cells are unknown. Therefore this paper will investigate the mechanism that is responsible for MSC derived anti-proliferation.

MATERIALS AND METHODS

Bone marrow samples were purchased from Stem Cell Technologies, USA whereby the samples were obtained in accordance with local ethics requirement. Bone marrow aspirates was diluted with 1X PBS (phosphate-buffered saline) and layered onto Ficoll-Paque PLUS (1.077 g/ml, Amersham Biosciences, Sweden) and centrifuged at 2000 rpm, 10°C for 20 min. Mononuclear cells (MNC) were collected, counted, and seeded in 25cm^2 vented culture flask (Nunc Brand Products) in MSC complete medium which consist of Dulbecco's modified Eagle's medium (DMEM) with high glucose and supplemented with 10% pre-selected fetal bovine serum. When the adherent cells achieved 80% to 90% of confluence, cells were trypsinised by using 0.05% trypsin-EDTA and further expanded in 75cm^2 flasks.

The immunophenotyping of the MSC was performed by using the FACSCalibur flow cytometer (Becton Dickinson). MSC were stained with the following fluorochrome conjugated monoclonal antibodies against CD105-PE, CD73-PE, CD90-PE, CD45-PerCP, HLAI-PECy5, HLAII-FITC, CD34-FITC, CD80-PE and CD86-APC for 30 minutes at 2-8°C and washed with PBS. Apoptotic cells were determined by

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AnnexinV-FITC positivity with an exclusion of PI staining. Cell cycle analysis was performed by evaluated intracellular proteins and DNA content by fluorescein isothiocynate (FITC) and propidium iodide (PI) dyes, respectively. Apoptosis and cell cycle assays were conducted at ratio of MSC: Tumour cells = 1:10 and the seeding density of tumour cells was 1.0 x 10^6 cells/ml.

All tumour cells were purchased from American Type Culture Collection (ATCC). BV173, a human B cell precursor leukaemia established from the peripheral blood of a patient with chronic myeloid leukaemia (CML) in blast crisis[10], Jurkat cell, a human T cell leukaemia established from the peripheral blood of a patient with acute lymphoblastic leukaemia (ALL)[11]. Both cells serve as model for lymphoid origin haematopoietic tumour cells.

Tumour cell proliferation assay was performed by seeding MSC at various ratios with a fixed numbers of tumour cells at 72 hours. A similar assay was also set by replacing MSC with supernatant collected from MSC culture. MSC supernatant was harvested from fully confluence MSC culture at 72 hours. The cells were pulsed with 3H-TdR (0.0185 MBq/well [0.5 µCi/well]) and incubated for 18 hours in 37°C and 5% CO2 humidified cell culture incubator. At the end of incubation, cells were harvested onto glass fibre filter mat (Perkin Elmer) using a 96 well plate automated cell harvester (HARVESTER 96 MACH III M, TOMTEC) and thymidine incorporation was measured by liquid scintillation spectroscopy on a beta counter (WALLAC Micro Beta Trilux, Perkin Elmer, Finland). The results were expressed in count per minute (CPM) or percentage of control proliferation.

Data were presented as the mean ± the standard deviation from 3 or more experiments. The statistical significance was determined by 2-tailed Student t test.

RESULTS

BM-MSC were morphologically heterogeneous at initial passages due to contamination of adherent haematopoietic cells especially macrophages and stromal cells. The formation of cluster like colonies of adherent cells was observed after 5-7 days of primary culture. Immunophenotyping of adherent cell from passage two revealed that, more than 90% of cells were positive for mesenchymal markers namely CD73, CD105, and CD90; did not express early or mature haematopoietic markers such as CD34 and CD45 (Figure 1a). In terms of immunological markers, MSC express MHC class I but neither express MHC class II, CD80 nor CD86 (Figure 1b).

When tumour cells were co-cultured in the presence or absence of BM-MSC at different ratios the tumour cell proliferation was inhibited in a dose dependent manner (Figure 2). MSC highest inhibitory activity was at ratio 1:5 (MSC:Tumour cells), inhibiting up to 80% of BV173 cell proliferation. However the inhibition of Jurkat cell proliferation was significantly lower when compared to BV173 cells. To examine whether the MSC-induced inhibition is mediated by section of soluble factors, proliferation assay was set up by physically separating MSC from tumour cells through a transwell system. The inhibitory effect of MSC is much pronounced when there is a direct cell to cell contact (Figure 3). This indicates that, MSC might require a certain extent of cell-to-cell contact interaction in order to fully exert their inhibitory activity. However, the potential role of soluble factors that may be responsible for this inhibition cannot be negated.

Apoptosis assay was performed using Annexin V and PI dye. Annexin V detects early apoptotic cells and PI binds to DNA content as an indication of membrane disintegrity. The result showed that MSC did not increase the percentage of Annexin V positive cells in tumour cells at any tested time points (Figure 4). However, MSC reduced the percentage of apoptotic and necrotic cell death compared to tumour cells alone, but with a statistically negligible significance.

In order to define the anti-proliferative effect of MSC on tumour cells, cell cycle analysis was utilised to elucidate the mechanism of MSC-mediated inhibition. Cell cycle analysis reveals that MSC did not inhibit the protein synthesis activity in G1 phase. However, PI staining indicates a substantial reduction in DNA synthesis in presence of MSC (Figure 5). Tumour cells were accumulated predominantly in G_0/G_1 phase in presence of MSC (BV173 vs. BV173 + MSC: 30.24% vs. 56.55%; Jurkat vs. Jurkat + MSC: 48.75% vs. 58.08%), thereby preventing cell cycle progression to S phase.

DISCUSSION

The effect of MSC on tumour cells has been mostly studied in solid tumour models notably breast cancer; however results were always contradictory[12-15]. The earliest study by Maestroni et al have shown that bone marrow stromal cells inhibit primary tumour growth and metastasis formation in mice transplanted with Lewis lung carcinoma or B16 melanoma[16]. Further supporting *in vivo* work from Nakamura *et al* (2004) has demonstrated that intra-tumoural injection of rat MSC at sites of tumour caused significant inhibition of rat 9L glioma and increased the survival of glioma bearing rats[17].

In this study, we have confined the anti-tumourigenic effect of MSC on two lymphoid origin haematopoietic tumour cells. Similar to other studies, MSC inhibit the tumour cell proliferation in a dose dependent manner[18,19]. We have also previously reported that MSC mediated inhibitory activity targets erythroid and myeloid origin haematopoietic tumour cell lines[9,20]. It is worth to mention that the inhibitory effect of MSC is highly confined to BV173 cells; however only a small degree of inhibition was noticed in Jurkat cells. Although the reason for certain cell types being more susceptible to MSC induced inhibition is unclear, however we could consider that the internal signalling pathways that govern cell cycle in various tumour cells might contribute to this discrepancy.

Although several studies are supporting our finding, however the mode of inhibition in our hand is profoundly different from others. Our results demonstrate that the *in vitro* anti-proliferative effect of MSC is mediated mainly by a direct cell-to-cell contact and lesser extent by secretion of soluble factors. On the contrary, Zhu and others showed that the

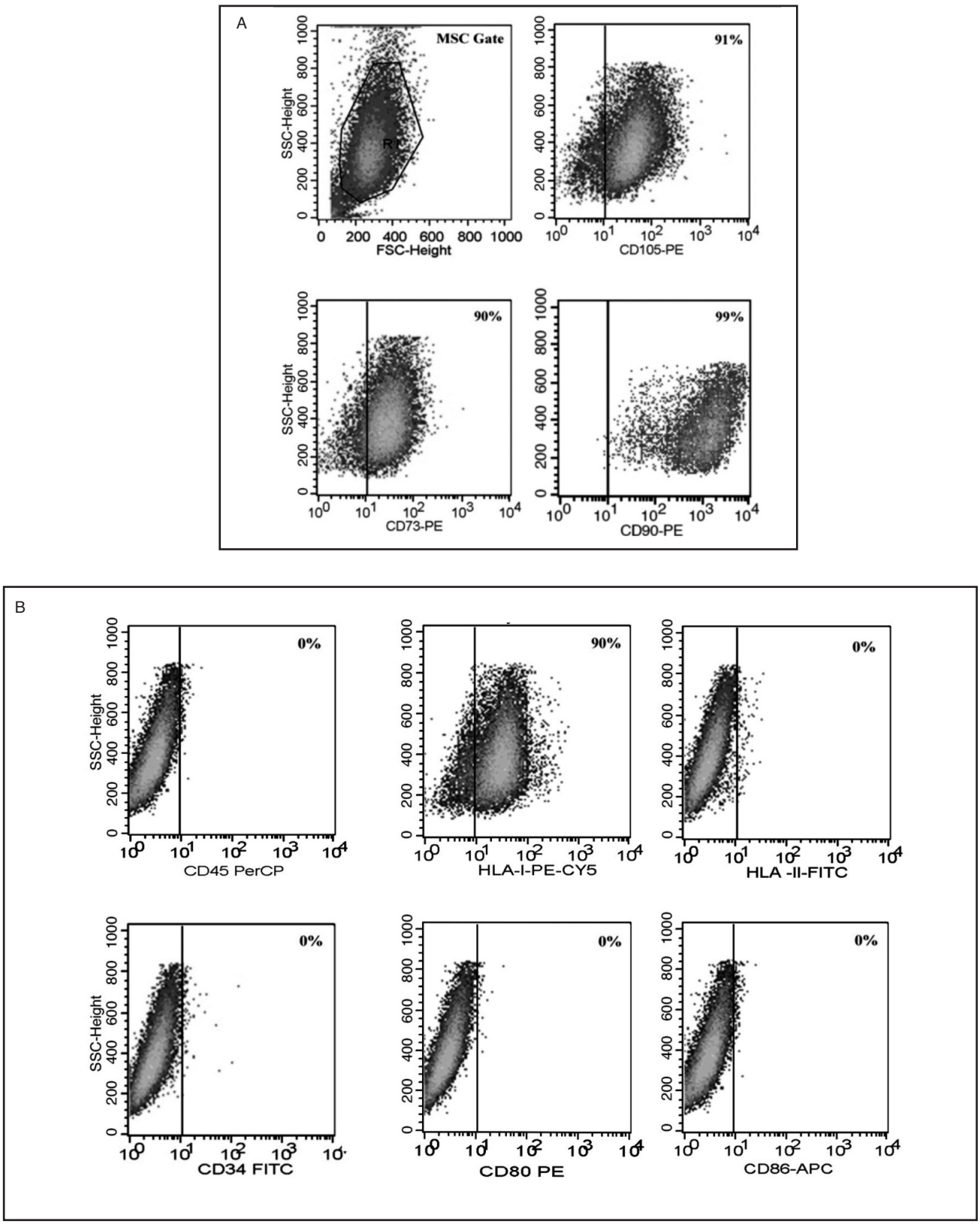


Fig. 1: Human adult bone marrow MSC express common mesenchymal markers (CD105, CD73 & CD90) [A]; negative for haematopoietic markers (CD34 & CD45) and lack of immunogenic markers (CD80, CD86 & HLA-II) [B].

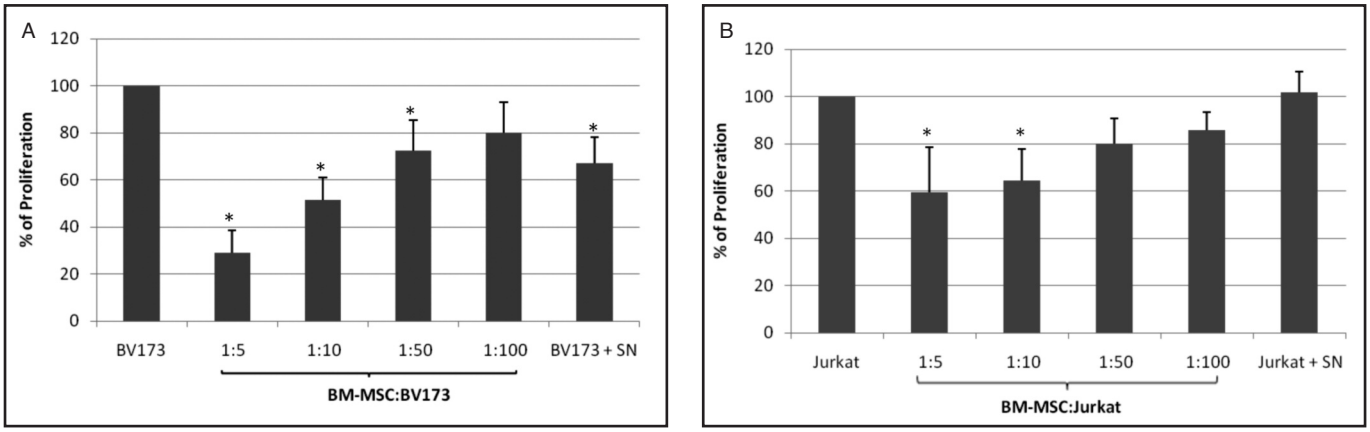


Fig. 2: MSC inhibits proliferation of BV173 (A) and Jurkat (B) cell lines in dose dependent manner. The results are a mean of five different experiments. Bars show SEM. [*] are statically significant ($p<0.05$) when compared with proliferation assay without MSC. SN-Supernatant.

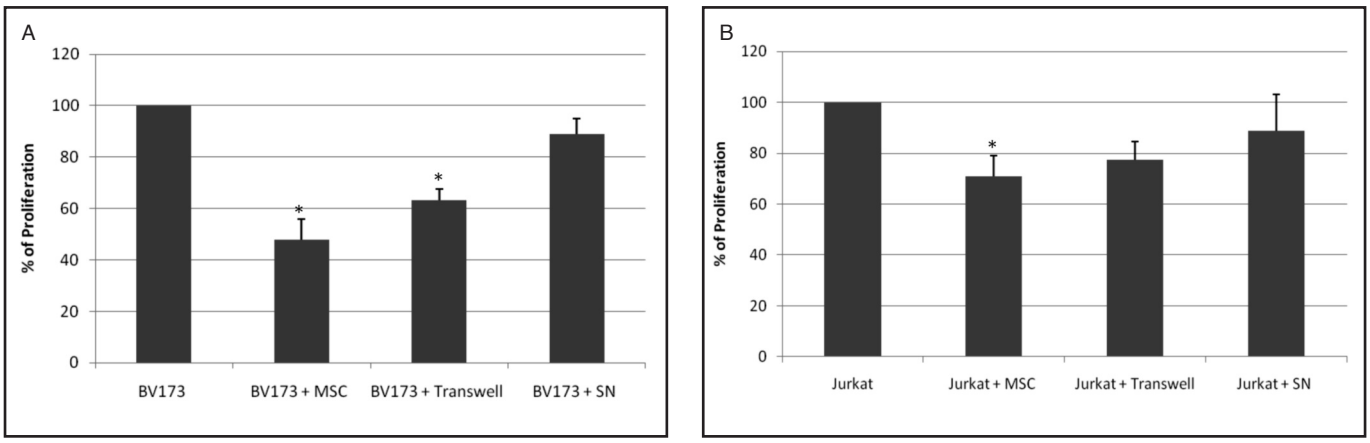


Fig. 3: The anti-proliferative activity exerted by MSC on BV173 (A) and Jurkat (B) cell lines is mainly due to cell to cell contact interaction. This result is mean of three different experiments and [*] indicate statistically significance ($p<0.05$) when compared with proliferation assay without MSC.

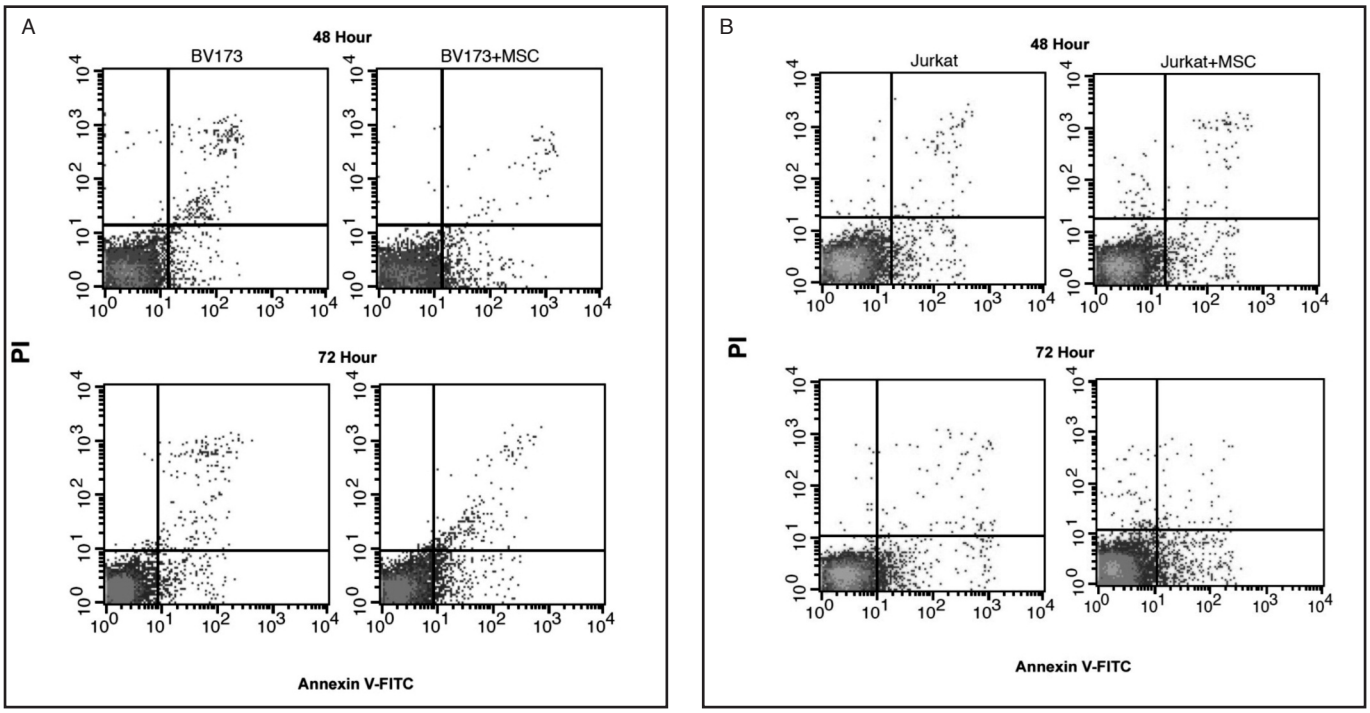
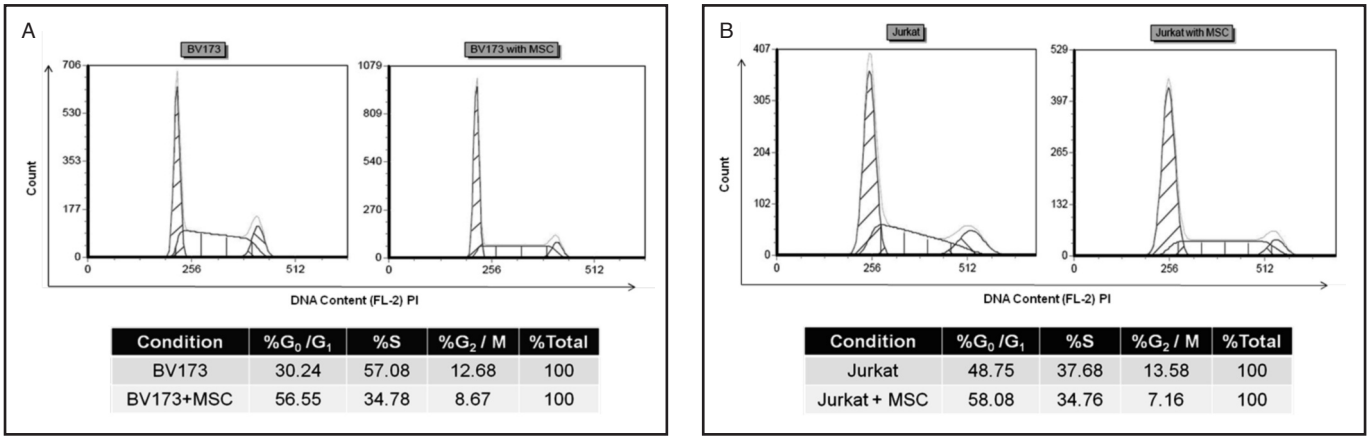


Fig. 4: MSC did not induce apoptosis in BV173 (A) and Jurkat (B) cell lines at 48 and 72 hours time points.



Condition	%G_0/G_1	%S	%G_2 / M	%Total
BV173	30.24	57.08	12.68	100
BV173+MSC	56.55	34.78	8.67	100

Condition	%G_0/G_1	%S	%G_2 / M	%Total
Jurkat	48.75	37.68	13.58	100
Jurkat + MSC	58.08	34.76	7.16	100

Fig. 5: MSC prevent tumour cell entry into S phase of cell cycle by arresting the cells in G0/G1 phase. This is a representative data of three different experiments.

inhibitory effect of MSC was achieved through secretion of a soluble factor[21,22]. However, we still could not negate the role of soluble factor in mediating anti-proliferation as transwell experiments indicate a noticeable degree of inhibition when MSC were physically separated from tumour cells.

To ascertain whether the inhibitory effect of MSC is through initiation of programmed cell death or necrosis, tumour cells were co-cultured with MSC at different time points for Annexin V expression analysis. However, the inhibition is neither due to apoptosis nor necrosis. Although MSC did not increase the percentage of early apoptotic cell population, however a slight reduction of apoptosis is notable in the presence of MSC. This demonstrates that MSC induced inhibition is not due to a killing process, but inflicting a direct control on cell cycle machinery of tumour cells.

Continuous activation of cell cycle is a vital requirement for the tumour cells to proliferate and metastases. Therefore, targeting cell cycle status would be our next investigation point whereby exploration of cell cycle phases and cell cycle check points will shed the light in finding the focal point of MSC-mediated inhibition. Our results show that the in vitro suppression of tumour cell proliferation by MSC is generally mediated by an arrest in G_0/G_1 phase cell cycle. G_0/G_1 phase of cell cycle is a doorway for the cells to enter cell division or cellular differentiation[23,24]. Completion of cell cycle requires synthesis of proteins (G_1 phase) which is necessary for executing the cell division process and also duplication of DNA (S phase) to equally produce two progenies. The cell cycle process is complete once the replicated nucleuses undergo cytokinesis (G_2/M phase) to form two distinct daughter cells. Any obstruction in these phases will potentially halt the cell division and lead to an arrest in proliferation[25,26]. The prominent cell cycle arrest in G_0/G_1 phase occurs in BV173 cells resulting in significantly lower cells in S phase. However, in lesser extent, Jurkat cells were also arrested indistinctly in G_0/G_1 phase but efficiently prevented from entering G_2/M phase. Although cell cycle machinery of both tumour cells were severely affected by MSC, the differences in phases involved in MSC-mediated inhibition suggests participation of different pathways of tumour cells proliferation despite their similar origin. An understanding of the mechanisms by which MSC inhibit the cell cycle progression may facilitate its further clinical development because this knowledge could lead to the identification of mechanisms based on biomarkers which can be potentially useful in future clinical trials. Although, considerable progress has been made towards the delineation of the signalling pathways responsible for MSC mediated cell cycle arrest, the mechanism by which this MSC-mediated inhibition of cell cycle progression is not fully understood.

CONCLUSION

Our work has confirmed the notion that MSC inhibit lymphoid origin tumour cell proliferation by inducing an arrest at the G_0/G_1 phase of cell cycle. This cell cycle inhibition might be due to activation or inhibition of various signalling pathways as the targets of cell cycle may vary based on individual cell lines. However, MSC-mediated anti-tumourigenic effect would be an ideal cell therapy to treat cancers. To realise this notion, an extensive investigation on MSC induced anti-proliferation against primary tumour cells and many established tumour cell lines is needed.

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