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ORIGINAL ARTICLE

The effects of mesenchymal stem cells on lymphoblastic leukemia cell proliferation

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Summary

Purpose: *Mesenchymal stem cells (MSCs) represent a new approach to the treatment of several neoplastic or non-neoplastic disorders. Their potential to repair damaged tissues through trans differentiation in conjunction with their immunomodulatory ability made them promising candidates for cell-based immunotherapy and regenerative medicine. In the present study, we aimed to determine the effects of MSCs on proliferation, apoptosis and gene expression profile of the acute lymphoblastic leukemia (ALL) cell line CCRF-CEM.*

Methods: *The experiments were performed after MSCs and CCRF-CEM cells were co-cultured for 72hrs. We analyzed the gene expression patterns to predict oncogenic pathway dysregulation in the cell groups by quantitative RT-PCR and immunohistochemical staining.*

Results: *Cell proliferation was significantly inhibited in co-cultured CCRF-CEM cells compared to the control. Furthermore, growth factors, p53, Bax and Caspase-9 expressions were increased and cell-signaling gene expressions decreased significantly. Despite increased levels of growth factors (CTGF, VEGF, FGF, EGFR), the increased apoptosis level was triggered by p53/ Bax.*

Conclusion: *In this study we have shown that human MSCs have inhibitory effect on their neighboring malignant leukemia cells.*

Key words: *acute lymphoblastic leukemia, apoptosis, CCRF-CEM cell line, co-culture, mesenchymal stem cells*

Introduction

Childhood leukemia represents about 35% of all childhood malignancies. Acute lymphoblastic leukemia (ALL) is one of the most common forms of childhood cancer, representing 80-85% of all leukemia cases [1]. MSCs play an important role in the regulation of hematopoiesis and engraftment of transplanted hematopoietic stem cells. Several studies have shown tumor stroma supporting effects of MSCs mainly in solid tumors including breast and gastric cancer. MSCs express growth factors, enhance tumor vessel formation and create tumor stem cell niches. Based on these characteristics, MSCs have several tumor growth promoting functions in the tumor microenvironment [2]. On the other hand, the effects of MSCs on leukemic cells are controversial. It was reported that MSCs might induce apoptosis in leukemia cells [3]. MSCs secrete various cytokines and chemokines that suppress proliferation of lymphocytes by arresting them at G0/G1 phase of the cell cycle as reported by several authors [4]. Therefore, the effects of MSCs in lymphocytic leukemia remain to be elucidated.

Over two decades, cell-based immunotherapy has become a major entity of chemoresistant leukemia treatment. The infusion of unmodified donor lymphocytes following relapse after allogeneic transplantation may be curative in patients with chronic myeloid leukemia (CML) and in some cases with acute myeloid leukemia (AML). However, this potentially curative role of cell-based immunotherapy has not been observed in patients with ALL [5]. These observations suggest that some tumor cells may escape from immune system

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by different molecular mechanisms. One mechanism is tumor cell escape from programmed cell death by expression of anti-apoptotic molecules or downregulation and mutation of pro-apoptotic molecules [6]. The balance between pro-apoptotic and anti-apoptotic BCL-2 (B cell leukemia/lymphoma-2) protein family members controls the mitochondrial apoptotic pathway and targeting this mechanism may have a therapeutic role in ALL [7]. In order to better understand how the MSCs affect leukemia cells, we studied the effects of MSCs on an ALL cell line CCRF-CEM. The proliferation rate, cytotoxicity, apoptosis and expression profiles of the genes that have very important functions in oncogenesis were investigated.

Methods

Cell lines

Bone marrow derived human MSCs were obtained from Millipore and grown according to manufacturer's protocol. They were cultured with Mesenchymal Stem Cell Expansion Medium (MSC-EM, Millipore, Darmstadt, Germany) supplemented with freshly added 8 ng/mL fibroblast growth factor-2 (FGF-2, Millipore). Nunclon™Δ certified polystyrene flasks were used for MSC culture experiments. MSCs were validated for high expression level of cell surface molecules (CD44, CD90, STRO-1) and for their absence of hematopoietic cell surface markers (CD14 and CD19). MSCs are also negative for the expression of the endothelial marker M-CAM (CD146). The cells have also been validated for their self-renewal and multi-lineage differentiation capacities. Human T lymphoblast ALL cell line CCRF-CEM was obtained from ATCC and cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and streptomycin (Bio Ind., Kibbutz Beit Haemek, Israel).

Preparation of the MSCs-conditioned medium

Passage 3-5 MSCs were used in all experiments. 2x106 cells were plated in T75 flask. Mesenchymal Stem Cell Expansion Medium (MSC-EM) was changed every two days and supplemented with freshly added FGF-2. At the third replacement, medium was collected, filtered through a 0.2 mm filter, and stored at –80°C as MSCs-conditioned medium (hMSCs-CM) [8]. At this stage MSCs were 80–90% confluent in the culture flask. MSC-EM was used as control.

Cytotoxicity assay

CCRF-CEM cell suspension was placed into 96-well plate at a density of $1x10^5$ cells in 100 µl RPMI-1640 medium per well, and 100 µl MSC-conditioned medium was added to each well. Control cells were grown in the 100 µl RPMI-1640 + 100 µl MSC-EM without FGF-2. After 24, 48, 72 and 96 hours of incubation WST (Roche Applied Science, Mannheim, Germany) reagent was added to each well and incubated for 1 h. Formazan formation was quantified spectrophotometrically at 450 nm using a micro plate reader (Thermo, Vantaa, Finland). All experiments were performed in triplicate.

Co-culture experiments

Polycarbonate cell inserts with 0.4 µm pore size were used for the co-culture experiments (Thermo). MSCs suspension (500µl, $4x10^5$ cells) was placed in the 6-well plate [8]. After 24-hr incubation the medium was aspirated, 2mL MSC-EM without FGF-2 were added into the wells, and then inserts were placed into each well. CCRF-CEM cell suspension ($4x10^5$ cells in 500µl RPMI) was added immediately into the cell inserts. For the control CCRF-CEM cells, 2ml MSC-EM without FGF-2 were added into an empty well, then an insert was placed into the well. CCRF-CEM cell suspension ($4x10^5$ cells in 500µl RPMI) were added into the cell insert and the medium was completed to 2ml. After 72-hr incubation, CCRF-CEM cells in the inserts of the co-culture systems were collected and counted with Cedex XS analyzer, which provides quantitative information about cell concentration and cell viability. The cell number in each insert was counted for three times, and the results were expressed as mean value ±SD.

Apoptosis assays

Cell death detection ELISA (Roche Applied Science, Mannheim, Germany) and Annexin V-FITC/PI apoptosis detection kits (Biovision, Research Products, Mountain View, CA, USA), were used for measurement of cell death. ELISA kit allows detection of mono and oligonucleosomes and measures apoptotic cell death. $1x10^5$ cells/ml were obtained from control and co-cultured CCRF-CEM wells. The cytoplasmic fraction was separated, diluted with incubation buffer (1:10) and immunoassay performed according to the manufacturer's protocol. At the end of the experiment, color development was quantified spectrophotometrically at 405 nm against substrate solution as blank, and enrichment factor was calculated according to the formula: mU of the sample (dying/dead cells)/mU against the control cells (mU=absorbance).

Annexin V-FITC/PI stained samples were analyzed with a fluorescence microscope (Olympus, Tokyo, Japan) to distinguish between viable (Annexin V-/PI-), apoptotic (Annexin V1+/PI-), and necrotic (Annexin V-/PI1+) cells.

Determination of differentially expressed genes by real-time ready array

Total RNA was prepared using the MagNA Pure LC RNA Isolation Kit (Roche Applied Science, Mannheim, Germany) after co-culture experiment of CCRF-

Table 1. Real-time ready array gene list

Symbol	*Other symbols*	*Name*
CTGF	CCN2, HCS24, IGFBP8, MGC102839, NOV2	Connective tissue growth factor
FGF1	AFGF, ECGF, ECGF-beta, ECGFA, ECGFB, FGF-alpha, FGFA, GLIO703, HBGF1	fibroblast growth factor 1 (acidic)
FGF18	FGF-18, ZFGF5	fibroblast growth factor 18
FGF2	BFGF, FGFB, HBGF-2	fibroblast growth factor 2 (basic)
FGF9	GAF, HBFG-9, MGC119914, MGC119915	fibroblast growth factor 9 (glia-activating factor)
TGFA	TFGA	transforming growth factor, alpha
TGFB2	MGC116892, TGF-beta2	transforming growth factor, beta 2
TGFBR2	AAT3, FAA3, LDS1B, LDS2B, MFS2, RIIC, TAAD2, TGFbeta-RII, TGFR-2	transforming growth factor, beta receptor II (70/80kDa)
THY1	CD90, FLJ33325	Thy-1 cell surface antigen
APAF1	APAF-1, CED4, DKFZp781B1145	Apoptotic peptidase activating factor 1
BAD	BBC2, BCL2L8	BCL2-associated agonist of cell death
BAK1	BAK, BAK-LIKE, BCL2L7, BCL2L7P1, CDN1, MGC117255, MGC3887	BCL2-antagonist/killer 1
BAX	BCL2L4	BCL2-associated X protein
BCL2	Bcl-2	B-cell CLL/lymphoma 2
BID	FP497, MGC15319, MGC42355	BH3 interacting domain death agonist
BIK	BIP1, BP4, NBK	BCL2-interacting killer (apoptosis-inducing)
CASP1	CARD17, ICE, IL1BC, INCA, P45	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
CASP10	ALPS2, FLICE2, MCH4	caspase 10, apoptosis-related cysteine peptidase
CASP12	CASP12P1	caspase 12 (gene/pseudogene)
CASP14	MGC119078, MGC119079, MICE	caspase 14, apoptosis-related cysteine peptidase
CASP2	CASP-2, ICH-1L, ICH-1L/1S, ICH1, NEDD2	caspase 2, apoptosis-related cysteine peptidase
CASP3	apopain, CPP32, CPP32B, SCA-1, Yama	caspase 3, apoptosis-related cysteine peptidase
CASP4	ICE(rel)II, ICEREL-II, ICH-2, Mih1/TX, TX	caspase 4, apoptosis-related cysteine peptidase
CASP5	ICE(rel)III, ICEREL-III, ICH-3, MGC141966	caspase 5, apoptosis- related cysteine peptidase
CASP6	MCH2	caspase 6, apoptosis- related cysteine peptidase
CASP7	CMH-1, ICE-LAP3, MCH3	caspase 7, apoptosis- related cysteine peptidase
CASP8	ALPS2B, CAP4, Casp-8, FLICE, FLJ17672, MACH, MCH5, MGC78473	caspase 8, apoptosis- related cysteine peptidase
CASP9	APAF-3, APAF3, CASPASE-9c, ICE-LAP6, MCH6	caspase 9, apoptosis- related cysteine peptidase
CYCS	CYC, HCS, THC4	cytochrome c, somatic
FAS	ALPS1A, APO-1, APT1, CD95, FAS1, FASTM, TNFRSF6	Fas (TNF receptor superfamily, member 6)
FASLG	APT1LG1, CD178, CD95L, FasL, TNFSF6	Fas ligand (TNF superfamily, member 6)
AKT1	AKT, MGC99656, PKB, PKB-ALPHA, PRKBA, RAC, RAC-ALPHA	v-akt murine thymomaviral oncogene homolog 1
AKT2	PKBB, PKBBETA, PRKBB, RAC-BETA	v-akt murine thymomaviral oncogene homolog 2
AKT3	DKFZp434N0250, PKB-GAMMA, PKBG, PRKBG, RAC-gamma, RAC-PK-gamma, STK-2	v-akt murine thymomaviral oncogene homolog 3 (protein kinase B, gamma)
EGFR	ERBB, ERBB1, HER1, mENA, PIG61	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, (avian)
EPS15	AF-1P, AF1P, MLLT5	epidermal growth factor receptor pathway substrate 15
EPS8		epidermal growth factor receptor pathway substrate 8
GAB2	KIAA0571	GRB2-associated binding protein 2

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GRB2	ASH, EGFRBP-GRB2, Grb3-3, MST084, MSTP084	growth factor receptor-bound protein 2
INPP5D	hp51CN, SHIP	inositol polyphosphate-5-phosphatase, 145kDa
MTOR	FLJ44809, FRAP, FRAP1, FRAP2, RAFT1, RAPT1	mechanistic target of rapamycin (serine/threonine kinase)
PIK-3C2A	CPK, DKFZp686L193, MGC142218, PI3-K-C2(ALPHA), PI3-K-C2A, PI3K-C2alpha	phosphoinositide-3-kinase, class 2, alpha polypeptide
PIK3CA	MGC142161, MGC142163, p110-alpha, PI3K	phosphoinositide-3-kinase, catalytic, alpha polypeptide
PTPN11	BPTP3, CFC, MGC14433, NS1, PTP-1D, PT-P2C, SH-PTP2, SH-PTP3, SHP-2, SHP2	protein tyrosine phosphatase, non-receptortype 11
STAT1	DKFZp686B04100, ISGF-3, STAT91	signal transducer and activator of transcription 1, 91kDa
STAT2	ISGF-3, MGC59816, P113, STAT113	signal transducer and activator of transcription 2, 113kDa
STAT3	APRF, FLJ20882, HIES, MGC16063	signal transducer and activator of transcription 3 (acute-phase response factor)
STAT4	SLEB11	signal transducer and activator of transcription 4
STAT6	D12S1644, IL-4-STAT, STAT6B, STAT6C	signal transducer and activator of transcription 6, interleukin-4 induced
CCNB1	CCNB	cyclin B1
CCNB2	HsT17299	cyclin B2
CCNE1	CCNE	cyclin E1
CCNE2	CYCE2	cyclin E2
CDC2	CDC28A, CDK1, DKFZp686L20222, MGC111195	cell division cycle 2, G1 to S and G2 to M
CDC25A	CDC25A2	cell division cycle 25 homolog A (S. pombe)
CDC25C	CDC25	cell division cycle 25 homolog C (S. pombe)
CDK2	p33(CDK2)	cyclin-dependent kinase 2
CDK-N1A	CAP20, CDKN1, CIP1, MDA-6, P21, p21CIP1, p21Cip1/Waf1, SDI1, WAF1	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CD-KN1B	CDKN4, KIP1, MEN1B, MEN4, P27KIP1	cyclin-dependent kinase inhibitor 1B (p27, Kip1)
CDK-N2A	ARF, CDK4I, CDKN2, CMM2, INK4, INK4a, MLM, MTS1, p14, p14ARF, p16, p16INK4, p16INK4a, p19, TP16	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
CD-KN2C	INK4C, p18, p18-INK4C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
CHEK1	CHK1	CHK1 checkpoint homolog (S. pombe)
CHEK2	bA444G7, CDS1, CHK2, HuCds1, LFS2, PP1425, RAD53	CHK2 checkpoint homolog (S. pombe)
SFN	YWHAS	stratifin
WEE1	DKFZp686I18166, FLJ16446, WEE1A, WEE-1hu	WEE1 homolog (S. pombe)
JAK1	JAK1A, JAK1B, JTK3	Janus kinase 1
CD44	CD44R, CDW44, CSPG8, ECMR-III, HCELL, IN, LHR, MC56, MDU2, MDU3, MGC10468, MIC4, MUTCH-I, Pgp1	CD44 molecule (Indian blood group)
TNF	DIF, TNF-alpha, TNFA, TNFSF2	tumor necrosis factor (TNF superfamily, member 2)
KRAS	C-K-RAS, K-RAS2A, K-RAS2B, K-RAS4A, K-RAS4B, KI-RAS, KRAS1, KRAS2, NS3, RASK2	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MAP2K1	MAPKK1, MEK1, MKK1, PRKMK1	mitogen-activated protein kinase 1
MAP2K2	FLJ26075, MAPKK2, MEK2, MKK2, PRK-MK2	mitogen-activated protein kinase 2
MAPK1	ERK, ERK2, ERT1, MAPK2, p38, p40, p41, p41mapk, P42MAPK, PRKM1, PRKM2	mitogen-activated protein kinase 1

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MAPK3	ERK1, HS44KDAP, HUMKER1A, MGC20180, p44erk1, p44mapk, PRKM3	mitogen-activated protein kinase 3
MAPK8	JNK, JNK1, JNK1A2, JNK21B1/2, PRKM8, SAPK1	mitogen-activated protein kinase 8
BRAF	B-RAF1, BRAF1, FLJ95109, MGC126806, MGC138284, RAFB1	v-raf murine sarcoma viral oncogene homolog B1
MAPK7	BMK1, ERK4, ERK5, PRKM7	mitogen-activated protein kinase 7
ACTB	PS1TP5BP1	actin, beta
G6PD	G6PD1	glucose-6-phosphate dehydrogenase
GAPDH	G3PD, GAPD, MGC88685	glyceraldehyde-3-phosphate dehydrogenase
JAK2	JTK10	Janus kinase 2
LCK	p56lck, pp58lck, YT16	lymphocyte-specific protein tyrosine kinase
NFKB2	LYT-10, LYT10, p52	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)
FOXO3	AF6q21, DKFZp781A0677, FKHRL1, FKHRL1P2, FOXO2, FOXO3A, MGC12739, MGC31925	Forkhead box O3
CBL	C-CBL, CBL2, RNF55	Cas-Br-M (murine) ecotropic retroviral transforming sequence
TP53	FLJ92943, LFS1, p53, TRP53	tumor protein p53
TP63	AIS, B(p51A), B(p51B), EEC3, KET, LMS, NBP, OFC8, p40, p51, p53CP, p63, p73H, p73L, RHS, SHFM4, TP53CP, TP53L, TP73L	tumor protein p63
TP73	P73	tumor protein p73
FIGF	VEGF-D, VEGFD	c-fos induced growth factor (vascular endothelial growth factor D)
FLT1	FLT, VEGFR1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/ vascular permeability factor receptor)
VEGFA	MGC70609, MVCD1, VEGF, VEGF-A, VPF	vascular endothelial growth factor A
VEGFC	Flt4-L, VRP	vascular endothelial growth factor C

CEM and MSCs. Ten µg of total RNA were reverse-transcribed with the transcriptor high fidelity cDNA synthesis kit (Roche Applied Science, Mannheim, Germany). A real-time ready custom array panel was designed for the quantification of differently expressed gene expressions by real-time PCR using the LightCycler 480 instrument (Table 1). Relative quantification of each sample with G6PDH (glucose-6-phosphate dehydrogenase), GADPH (glyceraldehyde-3-phosphate dehydrogenase) and Beta-actin housekeeping genes was achieved by using the instruments software.

Immunohistochemical analyses

Control and co-cultured CCRF-CEM cells embedded in paraffin blocks were cut into 2µm sections with microtome (Leica MR 2145, Wetzlar, Germany). Immunohistochemical analyses were performed using primary antibodies BCL-2, BAX, Caspase-3, Caspase-8, Caspase-9, p53 and VEGF, all obtained from Santa Cruz, Texas, USA and diluted at 1/300. In brief, the deparaffinization procedure was accomplished in xylene for 1 h. Rehydration was done in sequential 100, 95, 80, and 70% alcohol series for 2 min each. After leaving in distilled water for 5 min, the samples were delineated on the object slide, washed in phosphate buffered saline (PBS) for 10 min, and then left in trypsin for 15 min. The primary antibody was then applied in an incubator at 37°C and washed with PBS. Afterwards the biotinylated secondary antibody was applied and washed with PBS before incubating with the enzyme conjugate and 3,3-diaminobenzidine tetrahydrochloride (DAB). Subsequently, sections were stained with Mayer's hematoxylin (Zymed Laboratories, San Francisco, CA, USA) and mounted with entellan. All sections were examined and photographed with Olympus C-5050 digital camera at Olympus BX51 microscope. The positive ratios of immunohistochemical analyses were determined by counting the positive and negative cells in the cell groups.

Statistics

Statistical analyses of gene expressions were calculated by CLC Main Workbench software (Aarchus, Denmark). After normalization to the housekeeping genes, log2 transformation was performed to the expression values and fold change; FDR (False Discovery Rate) corrected p values were calculated. Statistical analysis of cytotoxicity assays and quantitative

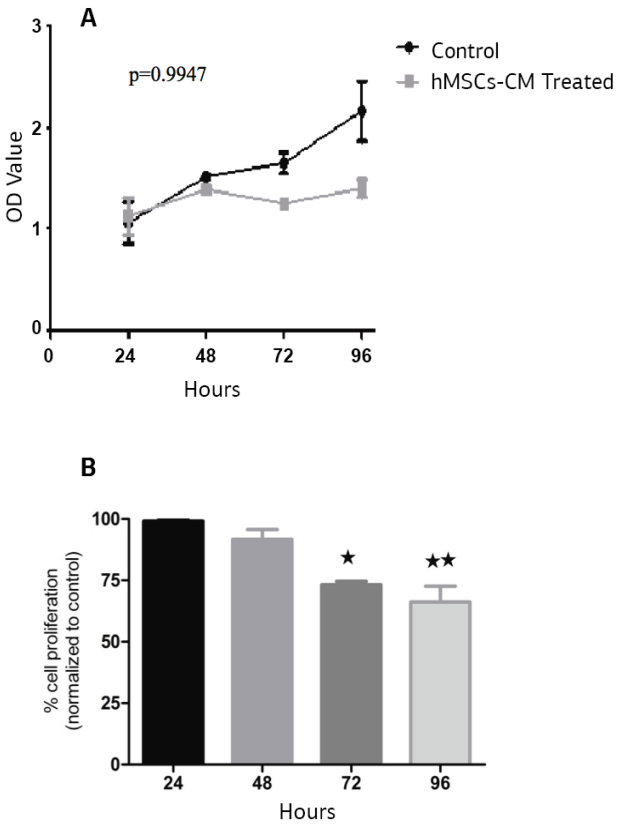

Figure 1. Effects of MSCs-conditioned medium on the CCRF-CEM cells viability. **A:** OD values of control and MSCs-CM treated groups of CCRF-CEM cells. **B:** The proliferation rate of MSCs-CM treated CCRF-CEM cells normalized to control. *p= 0.075, ** p=0.04

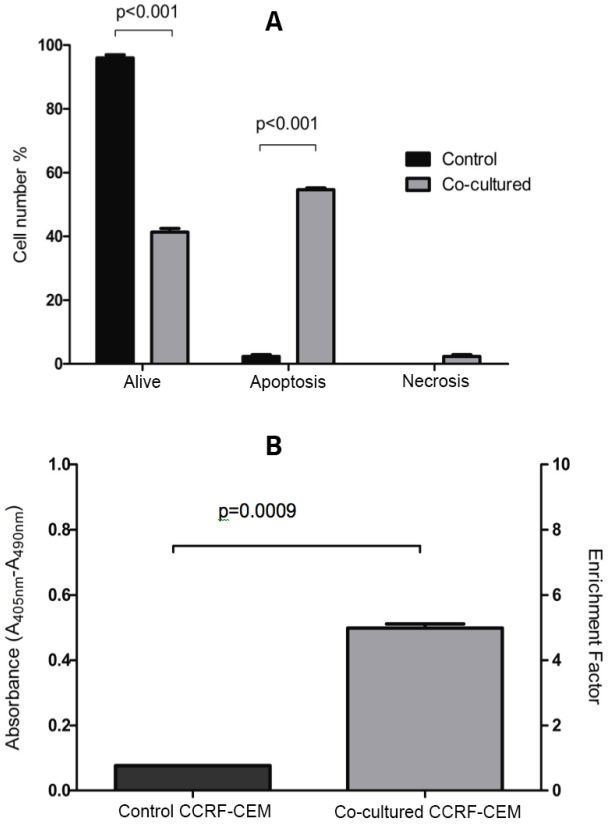

Figure 2. Effects of hMSCs on apoptosis. **A:** Annexin V-FITC/PI staining: Detection of apoptotic and necrotic cells by Annexin V-FITC staining in control and co-cultured CCRF-CEM cells. **B:** Cell death detection ELISA: Detection of nucleosomes in cytoplasm in control and co-cultured CCRF-CEM cells. CCRF-CEM cells were co-cultured with MSCs. After 72hrs CCRF-CEM cells were collected; the cytoplasmic fractions of the samples were obtained and prediluted 1:10 with incubation buffer, then tested with the immunoassay. Substrate reaction time: 10 min. Statistical significance was determined using Student's t test.

immunohistochemistry was performed by Wilcoxon Mann-Whitney non-parametric test using SPSS 13.0 software. The Student's t-test was used to test the probability of significant differences between samples. Statistical significance was set at $p<0.05$.

Results

MSCs-conditioned medium affects the viability of the CCRF-CEM cells

In order to study the effects of the soluble factors in the MSCs conditioned medium, CCRF-CEM cells were treated with MSCs-CM for 24, 48, 72, 96 hrs. The cell viability was evaluated by WST cytotoxicity assay (Figure 1A). Our results revealed that cell viability decreased as 8.29 ± 6.9, 26.77 ± 2.84 and 33.76 ± 11.09%, respectively at 48, 72 and 96 hrs compared to control (Figure 1B).

MSCs inhibit the CCRF-CEM cell proliferation in the co-culture system

In the co-culture systems, $4x10^5$ CCRF-CEM cells were cultured with the same count of MSCs. The cell count of co-cultured CCRF-CEM was significantly decreased compared to control ($0.72±0.12$ x10^5 vs $1.1±0.3$ x10^6; $p< 0.001$). These data confirms the presence of some factors produced from MSCs that exert an inhibitory effect on cell proliferation.

MSCs induce apoptosis in CCRF-CEM cells in the co-culture system

In order to examine the apoptotic effects of MSCs on CCRF-CEM, the cells were co-cultured for 72 hrs and apoptosis was evaluated using Annexin V-FITC/PI staining and cell death detection ELISA assay. Annexin V-FITC/PI staining results

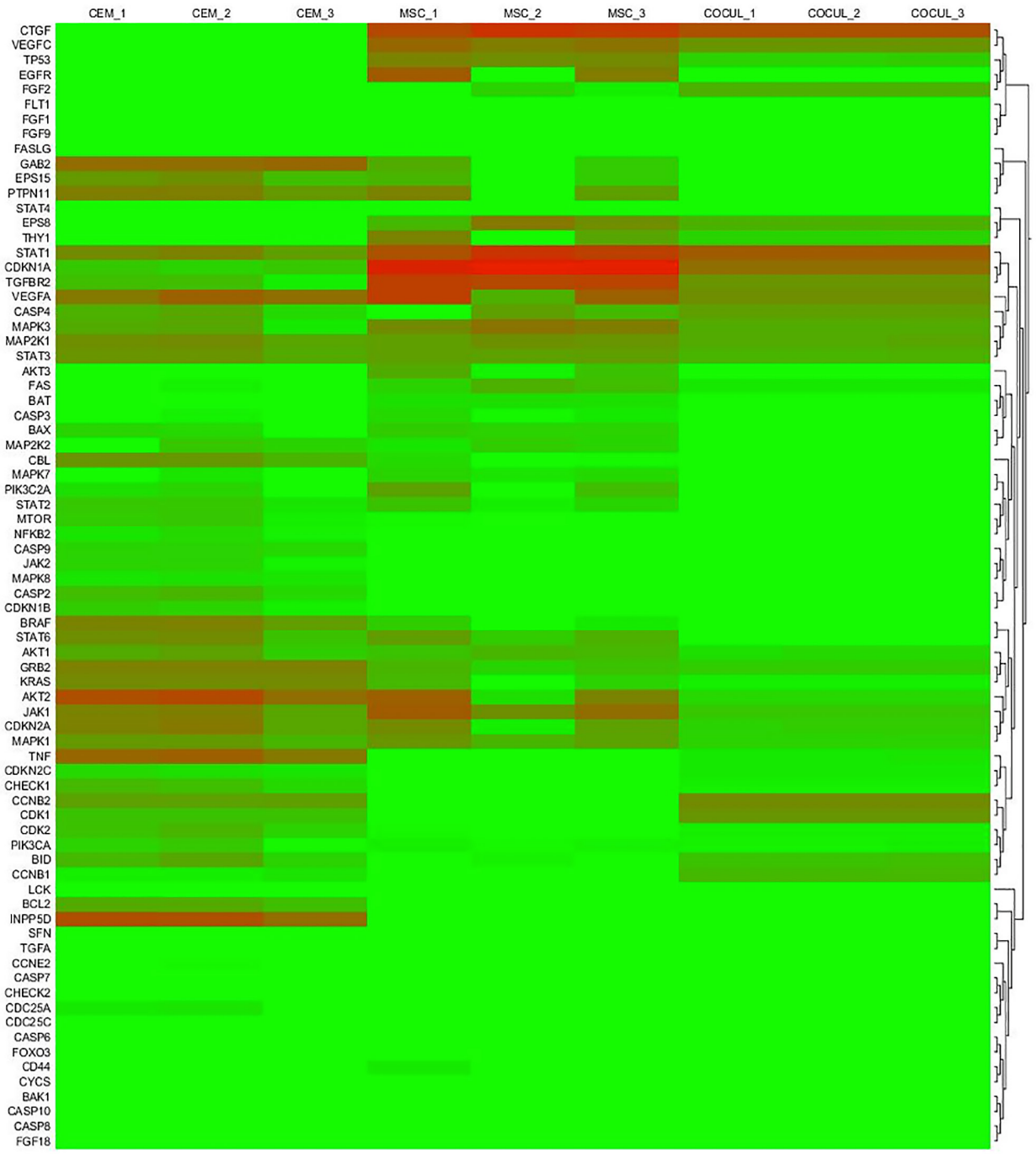

Figure 3. Hierarchical clustering of the gene expression profiles: Gene expression patterns of CCRF-CEM control, MSC and CCR-CEM co-cultured cell lines. Each sample was carried out in triplicate.

revealed that apoptosis rate increased significantly in co-cultured CCRF-CEM cells compared to control (2.38 vs 55.68%; $p<0.001$), as shown in Figure 2A. Also consistent with this result, we found that there was 6-fold increment for the enrichment factor in co-cultured CCRF-CEM cells ($p=0.0009$, Figure 2B). Cell viability and apoptosis assays clearly showed the obvious inhibiting effects of MSCs on CCRF-CEM cells.

MSCs dysregulate the gene expression profiles of the CCRF-CEM cells

Data obtained from gene expression analyses demonstrated that ALL cell line CCRF-CEM did not express any growth factors (Table 2). However, after co-culture with hMSCs, growth factors (CTGF, VEGFC, FGF1, FGF2, FGF9, EGFR, and EPS8) were expressed in CCRF-CEM cells (Figure 3). mRNA expression of tumor suppressor p53 gene increased by

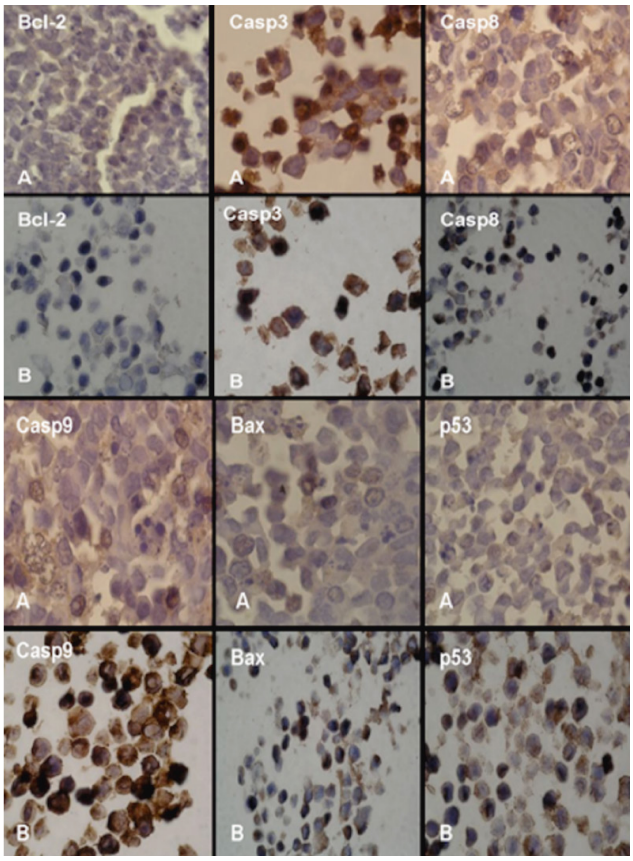

Figure 4. Immunohistochemical protein expressions in control and co-cultured CCRF-CEM cell groups. **A:** Control CCRF-CEM cells; **B:** Co-cultured CCRF-CEM cells with MSCs. The expressions of p53, Caspase-9 and Bax increased significantly after co-culture; on the contrary, Caspase-3, Caspase-8 and Bcl expressions decreased in co-cultured CCRF-CEM cells.

5.1 times in co-cultured CCRF-CEM cells ($p<0.001$, Table 2). However, mRNA expressions of adapter proteins (act for transmitting various signals in response to stimuli through cytokine and growth factor receptors, and T- and B-cell antigen receptors) decreased after co-culture treatments. GAB2 was determined as the most reduced gene (10.34-fold, $p<0.0001$) in co-cultured CCRF-CEM cells. A tyrosine phosphatase family member of PTPN11 mRNA expression was also reduced 8.51-fold ($p<0.0001$). The genes with decreased mRNA expression were WEE1 (-6.63-fold, $p=0.0001$), INPP5D (-3.57-fold, $p=0.0001$), AKT (-2.39-fold, $p=0.0007$), BRAF (-2.14-fold, $p=0.0002$), CBL (-2.37-fold, $p=0.0001$) and TNF (-2.11-fold, $p=0.0002$) as shown in Table 2 and Figure 3. Immunohistochemical analyses revealed that p53, Caspase-9 and Bax expressions were increased significantly after co-culture. However, Caspase-3, Caspase-8 and Bcl2 expressions were decreased in co-cultured CCRF-CEM cells. Immunohistochemical results are summarized in Figures 4 and 5.

Discussion

MSCs are non-hematopoietic progenitor cells capable to differentiate *in vitro* and *in vivo* into several mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, and fat [9]. They regulate lymphopoiesis and suppress the immune response. However, the biological mechanisms behind these observations remain unclear. Some authors claim cell-cell contacts, whereas others claim the role of cellular and soluble factors to

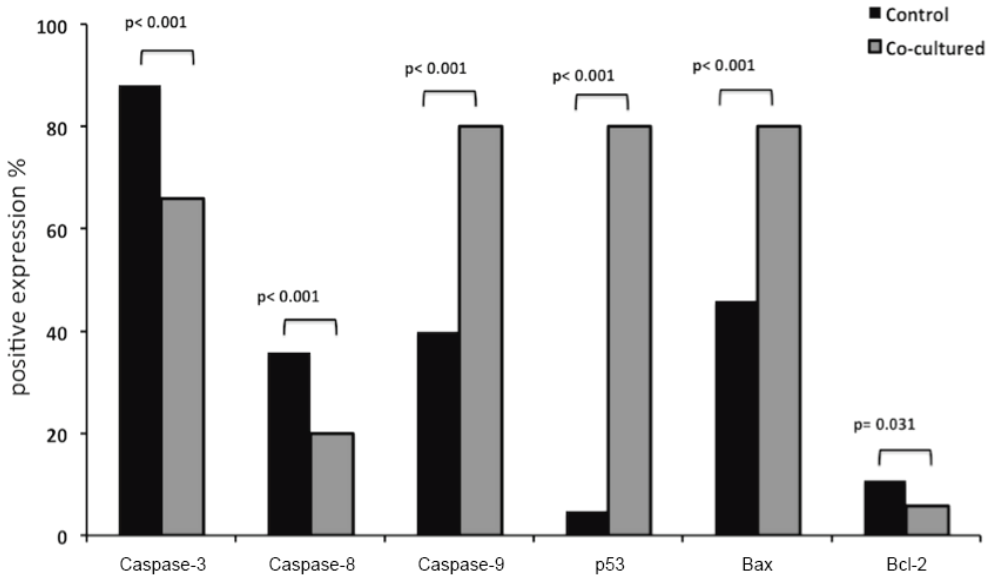

Figure 5. Data analysis of immunohistochemical protein expressions in control and co-cultured CCRF-CEM cell groups. Chi-square test revealed significant differences between the groups. Results are expressed as % positive expression of counted cells.

Table 2. List of genes that significantly increased and decreased in co-cultured CCRF-CEM cells compared to control

Gene	*Full name*	*Fold change**	*p value***	*Control CCRF-CEM Means*	*Co-cultured CCRF-CEM Means*
CTGF	Connective tissue growth factor	13.48	<0.0001	ND	0.15000
VEGFC	Vascular endothelial growth factor-C	7.76	<0.0001	ND	0.04000
FGF2	Fibroblast growth factor-2	6.50	<0.0001	ND	0.02000
TP53	Tumor protein 53	5.10	<0.0001	ND	0.01000
EGFR	Epidermal growth factor receptor	4.02	<0.0001	ND	0.00183
FGF1	Fibroblast growth factor-1	3.36	<0.0001	ND	0.00054
EPS8	Epidermal growth factor receptor pathway substrate-8	2.56	0.0012	0.00007	0.02000
FGF9	Fibroblast growth factor-9	2.43	<0.0001	ND	0.00003
BCL2	B cell leukemia/lymphoma-2	-2.07	<0.0001	0.02000	0.00032
TNF	Tumor necrosis factor	-2.11	0.0002	0.10000	0.00674
BRAF	v-raf murine sarcoma viral oncogene homolog-B1	-2.14	0.0002	0.05000	0.00137
CBL	Cbl proto-oncogene	-2.37	0.0001	0.03000	0.00023
AKT2	v-akt murine thymoma viral oncogene homolog	-2.39	0.0007	0.14000	0.00872
INPP5D	Inositol polyphosphate-5-phosphatase	-3.57	0.0001	0.13000	0.00061
FASLG	Fas ligand	-3.67	0.0001	0.00188	ND
WEE1	WEE1 homolog	-6.63	<0.0001	0.02000	ND
EPS15	Epidermal growth factor receptor pathway substrate 15	-7.11	<0.0001	0.03000	ND
PTPN11	Protein tyrosine phosphatase, non-receptor type-11	-8.51	<0.0001	0.05000	ND
GAB2	Grb2-associated binding protein	-10.34	<0.0001	0.09000	ND

*Values represent fold-changes of increased or decreased mRNA expressions. All values were normalized to three housekeeping genes and then log2 transformation was performed. **FDR corrected p value, ND: not determined.

contribute to MSCs-mediated inhibition of proliferation [10]. MSCs can inhibit the proliferation of lymphocytes stimulated by mitogens, antibodies, or alloantigens in a dose-dependent manner [4]. Plumas et al. reported that MSCs are unable to activate allogeneic T-cells even in the presence of T-cell growth factors [11]. Furthermore, MSCs inhibit T-cell proliferation by inducing apoptosis of activated T-cells but have no effect on resting T-cells. It was reported that MSCs express 2,3-dioxygenase (IDO), which is an enzyme catalyzing the conversion from tryptophan to kynurenine [12]. Soluble factors, such as IFN-γ, released during T-cell activation could induce IDO expression by MSCs and induction of apoptosis of activated T-cells [11]. Several studies have shown the involvement of cytokines, such as TNF-α, IFN-γ, TGF-β, IL-10, in MSCs-mediated immunosuppression [13]. MSCs produce cytokines, chemokines and growth factors, such as interleukins, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor [14].

MSCs have many properties that may induce malignant transformation and cancer initiation [15]. Recently, Momin et al. have indicated the oncogenic and tumor-supporting potential of MSCs and they suggested rigorous evaluations of their oncogenic potential [16]. Krampera and co-workers showed that the co-culture of B-ALL cells with hBM-MSCs significantly increased the number of surviving B-ALL cells, compared with that obtained by culturing B-ALL cells alone under the same conditions [17]. The difference in the results of Krampera's and our study could be due to the used MSCs and the lineage of ALL. Homegrown bone marrow-derived MSCs and B-lineage ALL cells were used in the Krampera's study. However, we used a standard bone marrow-derived MSCs and T-lineage ALL cell line CCRF-CEM.

Several other reports have suggested suppression of oncogenesis by MSCs [18]. A suppressor function of MSCs has also been observed for tumor cells of hematopoietic origin [19]. Our data supports the hypothesis that MSCs can inhibit the proliferation of ALL cells. The increased mRNA and protein expression of p53 was the potential factor for this inhibition. p53 is one of the most celebrated proteins because of its status as "guardian of the genome". It was reported that CCRF-CEM cell line has two heterozygous mutations located in the DNA binding domain of p53, thus its expression may have been functionally inactivated by two different point mutations [20]. G_2/M cell cycle arrest and apoptosis are induced in CCRF-CEM cells in p53-independent mechanisms [21]. Moreover, apoptosis was enhanced in the same cell line after transfection with wild-type p53 cDNA in comparison to controls [22]. Our results clearly showed that, p53 re-expression could be provided by MSCs derived factors in CCRF-CEM cells. BAX protein, which can be directly activated by p53, also increased significantly in the co-cultured CCRF-CEM cells and most likely increased the expressions of p53/Bax induced apoptosis in these cells.

It has been reported that effective cell death in B- and T-ALL cells depends on the presence of Caspase-8 for the majority of cytotoxic drugs routinely used in the treatment of leukemia [23]. Re-expression of epigenetically downregulated Caspase-8 represents a promising approach to increase the efficacy of antileukemic therapy. Our findings indicated that, although Caspase-8 expression decreased in both mRNA and protein level, cell proliferation was still inhibited. Caspase-3 is a protein that interacts with Caspase-8 and Caspase-9, and is activated in the apoptotic cells both by extrinsic and intrinsic pathways [24]. Surprisingly, in this study, after co-culture with MSCs, Caspase-3 expression was significantly decreased in CCRF-CEM cells. There is also some evidence for caspase-independent programmed cell death in the literature [25]. BAX and BAK proteins promote caspase activation and apoptosis, but they also kill cells independently of APAF-1 and downstream caspases, thus supporting our findings the p53/BAX-induced cell death [26].

Co-culture with MSCs changed the expression of some cell signaling and growth factor expression profile of CCRF-CEM cells. For instance, expression of GAB2 was the most reduced gene in this profile. GAB2 is a member of the GRB2-associated binding proteins gene family and binds SHP2 (PTPN11) tyrosine phosphatase and GRB2 adapter proteins. They act as adapters for transmitting various signals in response to stimuli through cytokine and growth factor receptors, and T- and B-cell antigen receptors [27]. Our results showed that GAB2 and PTPN11 expressions decreased -10 and -8 fold, respectively, in CCRF-CEM cells after co-culture ($p<0.0001$). PTPN11 encodes the protein tyrosine phosphatase SHP2 and positively regulates physiologic hematopoiesis [28]. Protein tyrosine phosphatases (PTPs) have critical roles in this regulation by controlling the functions of key receptors and intracellular signaling molecules in lymphocytes. In some cases, PTPs inhibit lymphocyte activation, whereas in others they promote it [29]. Our result was supported by a report in which negatively regulated PTPN11 in hematopoietic stem cells resulted in increased apoptosis [28].

The exact role of the different growth factors and cytokines in leukemic cell survival is not well known, but probably they form complex and dynamic networks in which leukemic blasts and stromal cells complement each other's cytokine expression, as shown in co-culture models of ALL [30]. Recently, some studies have shown that dysregulation of angiogenesis may lead to growth of leukemia and leukemia cells may proliferate like solid tumors [31]. MSCs secrete VEGF, TGF-β and matrix metalloproteinases, which have essential roles in angiogenesis. Proangiogenic properties of MSCs are favorable for tissue regeneration, but stimulating effects on tumor growth and metastasis were also revealed [32]. An increase of bone marrow vascularization in childhood ALL progression was published by Perez-Atayde et al. [33]. Bone marrow of these children had increased blood vessel content compared to normal counterparts, and it was suggested that leukemia might be angiogenesis-dependent. Although we did not find any expression of angiogenic factors in CCRF-CEM cells, they expressed CTGF, VEGF-C, FGF, EGFR and EPS8 after co-culture with MSCs. An increase in cell proliferation due to stimulation of growth factor secretion was expected. However, induction of apoptotic mechanisms and intracellular signaling pathways shifted the balance towards cell death.

In conclusion, we have shown that MSCs possess inhibitory effects on the proliferation of ALL cells in co-culture experiments. Apoptosis was induced by p53-Bax dependent manner and expression profile of genes associated with angiogenesis and cell signaling pathways was dysregulated

after co-culture of ALL cells with MSCs. Further studies are needed to delineate the role of MSCs in the modification of the biological behavior of ALL blasts which may have therapeutic implications, particularly after genetic manipulation.

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