Diverse marrow stromal cells protect CLL cells from spontaneous and drug-induced apoptosis: development of a reliable and reproducible system to assess stromal cell adhesion-mediated drug resistance

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ABSTRACT

Marrow stromal cells (MSC) provide important survival- and drug resistance signals to chronic lymphocytic leukemia (CLL) cells, but current models to analyze CLL-MSC interactions are heterogeneous. Therefore, we tested different human and murine MSC lines and primary human MSC for their ability to protect chronic lymphocytic leukemia (CLL) cells from spontaneous and drug-induced apoptosis. Our results show that both, human and murine MSC are equally effective in protecting CLL cells from fludarabine-induced apoptosis. This protective effect was sustained over a wide range of CLL-MSC ratios (5:1 to 100:1), and the levels of protection were reproducible in four different laboratories. Human and murine MSC also protected CLL cells from dexamethasone- and cyclophosphamide-induced apoptosis. This protection required cell-cell contact, and was virtually absent when CLL cells were separated from the MSC by micropore filters. Furthermore, MSC maintained Mcl-1 and protected CLL cells from spontaneous and fludarabine-induced Mcl-1 and PARP cleavage. Collectively, these studies define common denominators for CLL co-cultures with MSC. They also provide a reliable, validated tool for future investigations into the mechanism of MSC-CLL cross talk, and for drug testing in a more relevant fashion than the commonly used suspension cultures.
INTRODUCTION

With the establishment of more effective treatments for patients with chronic lymphocytic leukemia (CLL) over the last decade, complete remissions are no longer the exception\(^1\). Despite these major improvements in CLL treatment, we still consider CLL an incurable disease, because patients generally relapse from minimal residual disease (MRD)\(^2\). There is growing evidence suggesting that CLL cells are protected from conventional drugs in tissue microenvironments, such as the bone marrow and secondary lymphoid organs, with facilitation of residual disease that is drug resistant and ultimately paving the way to clonal evolution and relapses. The complex cellular and molecular context in the tissues, collectively referred to as the CLL microenvironment, provides signals for the expansion of the CLL clone, and for primary drug-resistance. This is largely dependent on direct contact between the malignant B cells and stromal cells\(^3\), and therefore has been designated as cell adhesion-mediated drug resistance (CAM-DR)\(^4\). Disrupting cross talk between leukemia cells and their milieu is an attractive novel but yet incompletely tested strategy for treating CLL. Appropriately, there is growing interest in understanding the biology of CLL-stroma cross talk in order to find ways to eliminate residual CLL cells that are “hiding” in stromal niches within the marrow and the lymphatic tissues.

Importantly, once CLL cells are removed from the \textit{in vivo} microenvironment and placed in suspension cultures without supportive stroma, they undergo spontaneous apoptosis, highlighting the importance of external signals from accessory cells\(^5\). Previous studies demonstrated that CLL cell co-cultures with different adherent cell types, collectively referred to as stromal cells, induce leukemia cell survival, migration, and drug resistance. These stromal cells include mesenchymal marrow stromal cells (MSC)\(^3, 6, 7\), CD68\(^+\) nurselike cells derived from monocytes\(^7-10\), and follicular dendritic cells\(^11\). Immunohistochemistry
revealed that in situ, αSMA⁺ mesenchymal stromal cells, the in vivo counterpart of MSC, are a dominant stromal cell population in the CLL microenvironment, which is in contrast to other B cell lymphomas, particularly high grade lymphomas, which harbor larger numbers of CD68⁺ hemangiogenic cells.

MSC regulate normal hematopoiesis by providing attachment sites and secreted or surface-bound growth factors that constitute the marrow microenvironment. During B cell development in the marrow, programmed cell death regulates B cell homeostasis by diverting a large fraction of immature B cells into an apoptotic death pathway to eliminate functionless or potentially harmful cells. Critical factors for the survival of selected B cells are interactions with MSC in the marrow microenvironment, expression of surface immunoglobulin molecules, and expression of apoptosis-regulatory proteins, such as Bcl-2. In patients with CLL, the marrow invariably is infiltrated with CLL B cells, and the pattern and extent of marrow infiltration correlates with clinical stage and prognosis. Because MSC are key regulators in normal B lymphopoiesis, and protect CLL cells from spontaneous or drug-induced apoptosis in vitro, it has been proposed that interactions with MSC play a key role in disease progression or resistance to therapy in CLL, other mature B cell malignancies, and acute lymphoblastic leukemia (ALL).

Previous studies to model the in vivo marrow microenvironment employed co-culture assays with various MSC of murine and human origins, and it has been of some concern that murine MSC may introduce confounding factors in these analyses. Also, it has been discussed whether primary MSC may be advantageous over MSC lines for studying CLL-MSC interactions. Furthermore, the reproducibility of MSC-based drug-resistance assays, a prerequisite for development of drugs that target CLL-MSC cross talk, has not yet been established. To address these questions, we explored the effects of different
human and murine MSC lines as well as primary human MSC on CLL cell \textit{in vitro} viability and drug-resistance. We established co-culture conditions for testing MSC-derived drug-resistance that were reproducible in different laboratories. Also, we explored in part the molecular mechanism related to the broad based stroma-derived drug resistance. This study therefore provides a basis for further investigation into the biology of MSC-CLL cell interactions, and for development and testing of new drugs or drug combinations that target MSC-derived drug-resistance.
MATERIALS AND METHODS

Cell purification, cell lines

After informed consent peripheral blood samples were obtained from patients fulfilling diagnostic and immunophenotypic criteria for B-cell chronic lymphocytic leukemia (CLL) at Leukemia Department, University of Texas, M.D. Anderson Cancer Center, Houston, TX; the Hematology Division, Mayo Clinic, Rochester, MN, and the Hematology Department, Medical University of Vienna, Austria. Patient consent for samples used in this study was obtained in accordance with the Declaration of Helsinki. Approval was obtained from the M.D. Anderson Cancer Center, Mayo Clinic and the Medical University of Vienna institutional review board for these studies. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll Paque (GE Healthcare, Uppsala, Sweden). Cells were used fresh or viably frozen in fetal bovine serum (FBS, SAFC Biosciences, Lenexa, KS) plus 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St.-Louis, MO) for storage in liquid nitrogen.

The murine stromal cell line M210B4 derived from (C57BL/6J×C3H/HeJ) F1 mouse was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The murine stromal cell lines KUM4 derived from bone marrow of a C3H/He mouse, KUSA-H1 derived from bone marrow of a C3H/He mouse and the murine stromal cell line ST2 derived from bone marrow of a BALB/c mouse were purchased from the Riken cell bank (Tsukuba, Japan). M210B4, KUM4, KUSA-H1, and ST2 cells were maintained in RPMI 1640 medium supplemented with 2.05 mM L-glutamine (HyClone, Logan, UT), 10% FBS (SAFC Biosciences, Lenexa, KS) and penicillin-streptomycin (Cellgro, Hemdon, VA).

The human mesenchymal cell line StromaNKtert derived from bone marrow and immortalized by human telomerase reverse transcriptase (hTERT)
and containing also exogene MFG-tsT-IRES-neo was purchased from Riken cell bank (Tsukuba, Japan). Cells were maintained in Minimum Essential Medium Eagle with Earl’s salts and L-glutamine (\(\alpha\)-MEM, HyClone, Logan, UT) supplemented with 12.5% FBS (SAFC Biosciences, Lenexa, KS), 12.5% Human Serum (Cellgro, Hemdon, VA), 1 \(\mu\)M hydrocortisone (Sigma-Aldrich, St.-Louis, MO) and 100 \(\mu\)M 2-mercaptoethanol (Sigma Aldrich, St.-Louis, MO). The human mesenchymal cell line UE6E7T-2 derived from bone marrow and immortalized by transformation with human papilloma viruses (HPV) HPV E6 and E7 as well as hTERT was purchased from Riken cell bank (Tsukuba, Japan). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (4500 mg/L) supplemented with 4 mM L-glutamine (HyClone, Logan, UT) and containing 10% FBS (SAFC Biosciences, Lenexa, KS) as well as penicillin-streptomycin (Cellgro, Hemdon, VA). The human mesenchymal cell line UCB408E6E7TERT-33 derived from umbilical cord blood and immortalized by transformation with HPV E6, HPV E7 and hTERT were purchased from Riken cell bank (Tsukuba, Japan). Cells were maintained in mesenchymal stem cell basal medium supplemented with mesenchymal stem cell growth medium single quotes (Poetics\textsuperscript{TM}, Lonza, Walkerville, MD, USA). Primary human MSC isolated from bone marrow of CLL patients were developed by us, as previously described\textsuperscript{27}. Cells were maintained in \(\alpha\)-MEM medium supplemented with L-glutamine, ribonucleosides and deoxyribonucleosides (HyClone, Logan, UT), and 20% FBS (SAFC Biosciences, Lenexa, KS). The morphology of each cell line is displayed in Fig. 1, and descriptions of each cell line are provided in the supplemental table Tab. S1.

\textit{Co-culture experiments}
For co-culture experiments stromal cells were seeded the day before the experiment onto 48 well-plates (Costar, Corning, NY) at a concentration of $5 \times 10^4$ cells/ml/well and incubated at $37^\circ C$ in 5% CO$_2$. After confirming the confluence of stromal layer by phase contrast microscopy, CLL cells were added onto the MSC layers at ratios between 5:1 to 100:1. For comparison, CLL cells were also cultured in suspension at a density of $5 \times 10^6$ cells/ml. For assessment of MSC-derived drug resistance, CLL cells were treated with 10 μM fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine, F-ara-A, Sigma-Aldrich, St.-Louis, MO), or 10 μM dexamethasone (Sigma-Aldrich, St.-Louis, MO), or 60 μM 4-hydroperoxycyclophosphamide (4-HC, kindly provided by Dr. M Colvin and Dr. S. Ludeman, Duke University, Durham, NC). At the indicated time points, CLL cells were collected by washing off the CLL cells, leaving the adherent stromal layer intact, and then assayed for cell viability. For experiments with 4-HC or 4-HC plus F-ara-A, CLL cells were incubated for 2 hours with or without 10 μM F-ara-A, followed by 45 minutes of incubation with 60 μM 4-HC and washing with fresh medium, as previously described$^{28}$. After that cells were placed in suspension culture or on top of MSC monolayer (KUSA-H1, Stroma NKTert) for 24 hours.

**Cell viability testing**

Determination of CLL cell viability after treatment with different concentrations of drugs was based on the analysis of mitochondrial transmembrane potential by 3,3′ dihexyloxacarbocyanine iodide (DiOC$_6$, Molecular probes, Invitrogen, Eugene, OR) and cell membrane permeability to propidium iodide (PI). Determination of CLL viability after 24, 48 and 72 hours treatment was performed as previously described$^7$. To differentiate CLL cells and stromal cells, we used a leukocyte gate that excluded large granular stromal cells, based on their forward- and side- scatter characteristics.
**MSC-CLL cell separation experiments**

In order to explore the impact of cell-to-cell contact in mediating drug resistance, we used micropore membranes to separate CLL and stromal cells. Cell culture inserts with pore sizes of 0.4 μm (BD Falcon™, Franklin Lakes, NJ) were used for non-contact co-cultures of CLL cells with MSC. Control CLL cells were cultured in contact co-cultures with MSC, or in suspension cultures without MSC. CLL cells then were treated with 10 μM F-ara-A, harvested after 24, 48 and 72 hours of incubation, and CLL cell viability was analyzed by flow cytometry.

**Immunoblotting**

Freshly isolated CLL cells were cultured in suspension or in contact co-cultures on confluent layers of seven different MSC cells lines at a ratio of 20:1 for 48 hours in the presence or absence of 10 μM F-ara-A. Control CLL cells in suspension were analyzed at 0 and 48 hours; for co-culture conditions, CLL samples were analyzed after 48 hours. After MSC co-culture, CLL cells were washed off the MSC layers and lysed on ice for 30 minutes in lysis buffer containing 25 mM HEPES, 300 mM NaCl, 1.5 mM MgCl₂, 0.5% sodium deoxycholate, 20 mM glycerophosphohate, 1% Triton X-100, 0.1% SDS, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, and protease inhibitor. Cells were centrifuged at 14000g for 15 minutes at 4°C, and supernatant was stored at -80°C until use. Protein content was determined using the detergent compatible (DC) protein assay kit, according to manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA). Aliquots (25 ug) of total cell protein were boiled with Laemmli sample buffer and loaded onto 8% to 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (GE Osmonics Labstore, Minnetonka, MN). Membranes were blocked for 1 hour in PBS-Tween containing
5% nonfat dried milk and incubated with primary antibodies either overnight or for 2 hours followed by species-specific horseradish peroxidase (HRP) conjugated secondary antibody (diluted 1:5000) for one hour. The blots were visualized by enhanced chemiluminescence according to manufacturer’s instructions (Pierce Biotechnology, Rockford, IL) and normalized to the actin levels in each extract. Membranes were probed at 4°C with the following primary antibodies: Mcl-1 antibodies (sc-819) from Santa-Cruz Biotechnology Inc., Santa Cruz, CA; PARP (Poly (ADP-ribose) polymerase) antibodies (BD Pharmingen International, San Diego, CA) and β-actin (Cell Signaling, Beverly, MA). Immunoreactive bands were visualized using peroxidase-conjugated secondary antibodies (GE Healthcare) and enhanced chemiluminescence detection system (Pierce Biotechnology, Rockford, IL). Cell viability was measured by flow cytometry for each time point and condition.

**Data analysis and statistics**

Results are shown as mean plus or minus standard error of mean (SEM) of at least 3 experiments each. For viability assays, we determined mean relative viabilities to account for variability in spontaneous apoptosis rates in different patients’ samples. We define the mean relative viability as the mean CLL cell viability of a particular sample (treated with drug X in the presence or absence of MSC at a certain time point), divided by the mean cell viability of the same sample at the same time point of control CLL cells, cultured in suspension culture. For statistical comparison between groups, the Student’s paired *t* test was used. Analyses were performed using GraphPad Prism 4 software for Macintosh (GraphPad Software, San Diego, CA). A *P* value less than 0.05 was considered as statistically significant. Flow cytometry data were analyzed using the FlowJo software (TreeStar, Ashland, OR).
RESULTS

Impact of different CLL-MSC ratios

In order to determine the impact of various CLL to MSC ratios on CLL cell survival, we explored a range of different ratios. Thus, we performed titration experiments and tested the following ratios of CLL to MSC cells: 5:1, 10:1, 20:1, 50:1 and 100:1. These conditions were tested in the presence and absence of F-ara-A in co-cultures with all seven MSC lines (stromal characteristics are shown in Fig. 1). Using 48 well plates, we found that lower ratios (5:1 and 10:1) did not allow for acquisition of sufficient CLL cell numbers by flow cytometry within a time frame that is feasible in larger scale experiments, and therefore we suggest using higher CLL-MSC ratios. Ratios of 20:1 or higher provided optimal conditions for timely and convenient viability testing. There was no significant difference in viability of CLL cells in presence or absence of 10 μM F-ara-A at 20:1, 50:1 and 100:1 ratios in all MSC cell lines tested (see supplemental Fig. S1), indicating optimal stroma-mediated protection within these cell ratios.

Murine and human MSC protect CLL cells from fludarabine-induced apoptosis

We next examined the effect of co-culture with MSC on the protection of CLL cells from spontaneous and drug-induced apoptosis. For these experiments, CLL cells were treated with 10 μM F-ara-A or left untreated and cultured in the presence or absence of MSC for 24, 48 and 72 hours and the percent viability was measured. As shown in Fig. 2, co-culture with murine MSC (M210B4, ST-2, KUM4 and KUSA-H1) protected CLL cells from both spontaneous and fludarabine-induced apoptosis at all time points, the mean relative viabilities (±SEM) and significances are summarized in the supplemental Table S3. Fig. 3 depicts the protective effect of human MSC (Stroma NKTerT, UE6E7T-2, UCB408E6E7 Tert33, hMSC from 2 patients) on the survival of CLL cells treated with 10μM F-ara-A. The mean relative viabilities (±SEM) and significances of CLL cell viabilities are summarized in the supplemental Table S3. These experiments demonstrate that murine MSC provide significant protection from F-ara-A-
induced apoptosis at 48 and 72 hours. Also, we did not notice any significant differences between different murine MSC lines (Fig. 2). In contrast, the levels of protection from F-ara-A-induced apoptosis at 48 and 72 hours with human MSC generally were lower when compared to murine MSC with the exception of Stroma-NKtert. Still, all human MSC provided significant protection from F-ara-A-induced apoptosis when compared to CLL cells treated with F-ara-A in suspension with the exception of the primary MSC sample hMSC#2 (Fig. 3).

**MSC protect CLL cells from dexamethasone-induced apoptosis**

We also tested the cytotoxicity of another chemotherapeutic agent, dexamethasone in co-cultures of CLL cells with murine and human MSC, using two cell lines that displayed high levels of protection in our experiments with F-ara-A. As depicted in Figure 4A, the mean viability of CLL cells treated with 10μM dexamethasone in absence of stromal cells was 74.3±6.8% after 24 hours, 51.9±11.7% after 48 hours and 45.3±12.3% after 72 hours. In presence of murine (KUSA H1) or human MSC (Stroma NKTert), the mean relative viabilities of CLL cell treated with 10μM dexamethasone were 117.3±7.0% or 117.4±7.1% at 24 hours, 110.2±12.0% or 137.2±10.3% at 48 hours, and 125.4±13.6% or 139.7±9.3% at 72 hours, respectively. Results are presented as the mean (±SEM) relative viability, n=5. These data indicate that murine and human MSC provide significant and relatively equivalent protection from dexamethasone induced apoptosis to CLL cells at all time points.

**Combination of 4-HC with F-ara-A partially overcome MSC-derived drug resistance in CLL**

To explore whether cell-adhesion drug resistance applies to other drugs commonly used for CLL treatment, we examined the cytotoxicity of 4-hydroperoxycyclophosphamide (4-HC), the bioreactive form of cyclophosphamide, alone or in combination with F-ara-A in co-cultures of CLL cells with murine and human MSC. CLL cells were treated with F-ara A, 4-HC or combination of these drugs with or without MSC, and viabilities were determined.
at 24 hours. As displayed in Figure 5, viability of CLL cells in suspension after treatment with 10μM F-ara-A was 80.7±3.4%, 7.4±2.5% after treatment with 60μM 4-HC and 4.6±2.0% after treatment with 10μM F-ara-A plus 60μM 4-HC. Co-culture of CLL cells with KUSA H1 or Stroma NK Tert increased the relative viability of CLL cells to 126.5±4.8% or 137.7±6.2% in the presence of F-ara-A; 23.1±4.1% or 44.4±5.9% after 4-HC and 12.6±3.9% or 18.5±5.1% after combination of 4-HC with F-ara-A. Results are presented as the mean±SEM relative to the viability of untreated controls (n=8). We conclude that presence of MSC improved CLL cell viability after treatment with 4-HC, or 4-HC plus F-ara-A when compared to suspension controls. While F-ara-A had no significant effect on CLL cell viability in MSC co-cultures at this time point, the combination of 4-HC plus F-ara-A induced significantly higher levels of apoptosis than 4-HC alone. This indicates that this mechanism-based combination of a nucleoside analogue with an alkylating agent with an alkylating agent is also active in CLL-MSC co-cultures.

**MSC-derived drug resistance in CLL cells is dependent on direct cell-to-cell contact**

We investigated the role of direct cell-to-cell contact in MSC-derived drug resistance by using micropore inserts to separate MSC and CLL cells. As shown in Figure 6A, the mean viabilities of CLL cells treated with 10μM F-ara-A and cultured in suspension, in presence of KUSA H1, or separated from KUSA H1 by inserts was 76.6±3.4%, 139.2±10.5%, or 79.3±3.6%, respectively at 24 hours, 28.6±6.9%, 110.5±16.4%, or 38.3±7.0%, at 48 hours, and 8.7±4.0%, 74.6±16.3%, or 14.1±4.4% at 72 hours. As displayed in Figure 6B, the mean viabilities of CLL cells treated with 10μM F-ara-A after 24 hours was 75.5±4.0% in suspension, 116.5±4.8% in contact co-cultures with Stroma NK Tert, and 78.3±4.7% when separated from StromaNK Tert by inserts. After 48 hours the mean viabilities were 26.5±6.5%, 87.3±19.7%, or 28.4±6.1% for these respective conditions, and 8.5±4.4%, 90.2±17.7%, or 9.9±3.4% after 72 hours. Results are
the mean±SEM relative viability of untreated controls in suspension (n=6). There were no significant difference in CLL cell viabilities between CLL suspension cultures and MSC non-contact co-cultures with culture inserts, indicating that CLL cell contact to MSC is a major contributing factor, and that soluble factors have a minor role in MSC-derived protection from F-ara-A-induced apoptosis.

Validation of MSC-CLL co-culture protocol in different laboratories

Three other laboratories in different regions were used to validate the MSC-CLL co-culture protocol in terms of the MSC-derived drug resistance described above. As displayed in Fig. 2S, all sites confirmed the efficacy of MSC in protecting CLL cells from spontaneous and F-ara-A-induced apoptosis. Site#1 (Medical University of Vienna, Austria) performed the experiments with M210B4 cells, and found that the mean viability of CLL cells in suspension treated with 10μM F-ara-A and was 84.3±4.1%, 37.7±4.9% and 20.5±3.7% after 24, 48 and 72 hours, respectively. When co-cultured with M210B4 without F-ara-A the mean viability was 125.3±8.3%, 143.7±15.1% and 140.8±14.7% at indicated time points. Adding the F-ara-A to co-culture conditions resulted in decrease of the mean viability to 114.9±5.1% after 24 hours (p<0.02 in comparison to CLL cells treated and cultured in suspension), 107.8±8.8% after 48 hours (p<0.001) and 85.3±6.8% after 72 hours (p<0.0007). Site#2 (Experimental Therapeutics Department, The University of Texas M.D. Anderson Cancer Center, TX) performed the experiments with KUSA H1 cells. The mean viabilities of CLL cells treated with 10μM F-ara-A and cultured in suspension was 77.5±6.3% at 24 hours, 38.1±10.5% at 48 hours and 13.8±9.6% at 72 hours. When cultured on KUSA H1 cells without F-ara-A, the mean viabilities of CLL cells were 139.2±4.0%, 151.0±4.3%, and 153.9±8.9% at these time points, and 124.2±5.5%, 98.7±10.8%, and 83.2±19.9% in the presence of F-ara-A plus MSC. Results are presented as the mean plus or minus SEM relative to the viability of untreated controls (n=5). Site#3 (Division of Hematology, Mayo Clinic, Rochester, MN) tested this protocol in co-cultures with M210B4. The mean viabilities of CLL
cells in suspension treated with 10 μM F-ara-A were 94.3±1.1% at 24 hours, 50.9±12.1% at 48 hours and 22.7±12.3% at 72 hours. The addition of F-ara-A to CLL-MSC co-cultures resulted in mean viabilities of 93.7±1.7% after 24 hours, 79.7±6.6% after 48 hours and 81.3±10.3% after 72 hours. When co-cultured with MSC without F-ara-A, the mean viabilities were 94.4±2.4%, 88.2±11.1% and 96.6±12.6% at these time points. Results are presented as the mean plus or minus SEM relative to the viability of untreated controls (n=4).

**MSC protect CLL cells from spontaneous and F-ara-A-induced apoptosis: effects on Mcl-1 and PARP**

In order to explore and define the molecular pathways of MSC-derived protection of CLL cells from spontaneous and F-ara-A induced apoptosis, we tested protein levels of Mcl-1 and PARP. These two proteins are known to change rapidly when CLL B cells interact with certain stromal cells, but the simultaneous testing of these two molecules with our spectrum of stromal cell lines has not been done. Thus CLL cells were cultured with 7 different MSC cell lines in the presence or absence of 10 μM F-ara-A, or in suspension. Culture in suspension for 48 h (second lane from the left) resulted in spontaneous apoptosis (47% viability in Fig. 7A, and 81% viability in Fig. 7B), and was accompanied by the generation of cleaved fragments of Mcl-1 (24 kD) and PARP (85 kD). Co-culture with MSC induced a significant increase of Mcl-1 expression in CLL cells after 48 hours of incubation in comparison to control in suspension, except for KUSA H1. Treatment with F-ara-A resulted in significant decreases in CLL cell viabilities in suspension after 48 hours, accompanied by a virtually complete cleavage of Mcl-1 and PARP (fifth lane from the left). Noticeably, these decreases in full-length Mcl-1 and PARP correlated well with the viability data collected by flow cytometry, as displayed in Fig. 7. In contrast, co-culture with MSC resulted in decreased or abrogated spontaneous and F-ara-A-induced cleavage of Mcl-1 and PARP in CLL cells. These results suggest that induction and/or maintenance of Mcl-1 by MSC plays an important role in protection of CLL cells from spontaneous and drug-induced apoptosis.
Discussion

The importance of the marrow microenvironment for protection of CLL and other leukemia cells from drug-induced apoptosis is increasingly recognized, based upon a series of in vitro and in vivo studies published over the last decade (reviewed in\textsuperscript{5, 22}). These studies defined the importance of accessory cells, in particularly of MSC\textsuperscript{3, 7, 23, 29} for providing survival, growth and drug resistance signals, which may explain why the marrow is a preferential site for minimal residual disease\textsuperscript{2, 30} and relapses even after extremely effective conventional treatments\textsuperscript{22}. Therefore, disrupting the cross talk between CLL cells and their milieu is an attractive and needed novel strategy. For this to be done in a meaningful way it will be necessary to devise testable \textit{in vitro} models. We have begun this process by attempting to standardize \textit{in vitro} culture conditions where we are examining stromal/CLL cell interactions. Thus we systematically examined a series of murine and human MSC, both cell lines and primary cells, for their capacity to support CLL cells and to protect them from drug-induced apoptosis. We found that both, murine and human MSC layers are highly effective in protecting CLL cells over a wide range of CLL-MSC ratios. This primary drug resistance mechanism, also called CAM-DR, is increasingly recognized as an key mechanism accounting for residual disease and relapses after conventional treatments in hematopoietic\textsuperscript{31} and other malignancies\textsuperscript{32}. These findings also suggest that the exact match between species is not critical for CLL cell protection. Indeed, we noticed that the levels of protection by murine MSC were more homogenous and higher in some cases, when compared to human MSC, with two human cell lines and primary human MSC displaying comparably less protective effects from F-ara-A (Fig. 3).

These findings parallel previous reports on the importance of MSC for hematopoietic progenitor cell maintenance and expansion in vitro. In the long-
term culture (LTC) systems, stromal feeder layers support the proliferation and
differentiation of hematopoietic progenitors in the absence of added cytokines.
Originally, these cultures were established from non-separated marrow cells\textsuperscript{33},
but subsequent studies established that adherent mesenchymal fibroblast lines
from different tissues and species can provide the same supportive function\textsuperscript{34}.
Previous reports comparing the capacity of different human and murine stromal
cells to support hematopoietic (long-term culture initiating cells [LTC-IC]) or B cell
progenitors in vitro demonstrated that murine MSC are working as well as human
MSC\textsuperscript{34-37}, or even better\textsuperscript{38}. The protective effect of MSC was largely dependent
upon direct cell-cell contact between the leukemia cells and the MSC, and
separation of the cells by micropore filters abrogated the protective function of
MSC (Fig. 6). This is in accordance with earlier notions that surface molecules
and surface-bound growth factors are the key communication factors involved in
MSC-derived survival signals\textsuperscript{3, 4, 13}. This, however, does not exclude the activity
of diffusible factors; for example, the chemokine CXCL12, which is generally
regarded as a secreted factor, can be sequestered and retained on the surface of
MSC by glycosaminoglycans and proteoglycans, increasing their local
concentration and availability\textsuperscript{39}.

We confirmed the reproducibility of these assays in four different
laboratories (Fig. S2). This broad based interactive approach for validation of
stromal cell support of CLL B cell survival will now allow us to move forward with
more systematic analyses including which classes of drugs are subject to
significant MSC-derived drug resistance, and importantly towards development of
drug combinations that account for MSC-derived drug resistance. The data
presented in this report demonstrate that MSC protect CLL cells from F-ara-A-,
dexamethasone-, and 4-HC-induced apoptosis. Also, our data indicate that drug
combinations (F-ara-A plus 4-HC) co-operate in inducing CLL cell apoptosis and
can, at least partially, overcome MSC-derived drug resistance, a finding that is in accordance with and helps to explain the clinical activity of this drug combination. Some examples of novel treatment approaches to overcome MSC-derived drug resistance already have been explored in CLL-MSC co-cultures. We reported that AT-101 induced apoptosis in CLL cells as equally as in the presence of MSC and down-regulated endogenous and MSC-induced Mcl-1. CXCR4 antagonists, which disrupt migration and adhesion of CLL cells to MSC, sensitized CLL cell to F-ara-A-induced apoptosis. Moreover, antagonists of phosphoinositide 3-kinases (PI3K) can overcome MSC-derived drug resistance. Cyclopamine, a hedgehog (Hh) signaling inhibitor, blocked MSC-induced survival of CLL cells, suggesting a role for Hh signaling in MSC-derived survival in CLL. Finally we have also shown that the timing and combination of epigallocatechin and curcumin can overcome stromal mediated drug resistance of CLL B cells.

Our molecular studies revealed that different MSC (human and murine) induced and effectively maintained Mcl-1 levels in CLL cells, and protected CLL cells from spontaneous and F-ara-A-induced apoptosis. Maintenance of intact, non-cleaved Mcl-1 was paralleled by high CLL cell viabilities (see Fig. 7), suggesting that Mcl-1 is critical for CLL cell viability in MSC co-cultures. Among the Bcl-2 family members, Mcl-1 has emerged as one of the most relevant antiapoptotic proteins in normal and malignant B cells. Mcl-1 is an early-response gene that functions as a modulator of cell viability. Mcl-1 can undergo rapid upregulation as well as downregulation (within 1-3 hours), which allows Mcl-1 to provide an acute protective function from apoptosis induced by various factors, including DNA damage, growth factor withdrawal, and treatment with cytotoxic agents. Disappearance of Mcl-1 is associated with the onset of apoptosis and is caused by the combination of synthesis blockage and
proteasomal degradation\textsuperscript{50, 54}. Cleavage of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) is another early marker of chemotherapy-induced apoptosis\textsuperscript{55}. The mechanism through which MSC maintain Mcl-1 in CLL cells even in the presence of F-ara-A are currently unknown. However, activation of CXCR4 chemokine receptors by its ligand, CXCL12 (stromal cell-derived factor-1/SDF-1) secreted constitutively by MSC, is a possible mechanism, given the earlier observation of Mcl-1 induction by CXCL12 in hematopoietic cells\textsuperscript{56}. Another study reported that B cell-activating factor of the tumor necrosis factor (TNF) family (BAFF) and a proliferation-inducing ligand (APRIL), but not CXCL12, induced Mcl-1 expression in CLL cells\textsuperscript{8}. In addition we have shown that vascular endothelial growth factor (VEGF), released by stromal cells, also can upregulate Mcl-1 in CLL cells\textsuperscript{27, 57, 58}. Recent studies indicate that Mcl-1 function and regulation is highly complex, integrating several proapoptotic and the prosurvival pathways, such as JNK and AKT\textsuperscript{54} and regulation by microRNAs\textsuperscript{59}. Ongoing studies in our laboratory, in which we determine gene expression changes induced in CLL cells by contact co-culture with different MSC, may help to further dissect out the critical pathways in CLL-MSC cross talk.

First trials that are directly targeting the CLL microenvironment are now entering the clinical stage. These trials are largely based upon data from \textit{in vitro} models that allowed us to define key pathways, such as the CXCR4-CXCL12 axis\textsuperscript{42, 60}, VLA-4 adhesion molecules and their respective stromal ligands (VCAM-1, fibronectin)\textsuperscript{4, 61}, the phosphoinositide 3-kinases (PI3K)\textsuperscript{43, 60}, and the spleen tyrosine kinase (Syk)\textsuperscript{62}. The model system described in this paper will help us to move forward with a more standardized approach for testing new and established drugs via \textit{in vitro} models that resemble the marrow microenvironment. The \textit{in vivo} validation\textsuperscript{61, 63, 64} of targets identified in these models, and the association of these findings with the clinical progress in this
area emphasize that co-culture models are now and will remain an indispensable tool for discovery and dissection of the tumor microenvironment in CLL and beyond.

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Authorship
A.K. performed the experiments, analyzed the data, designed the figures and wrote the paper with J.B., K.B. performed the western blots with A.K. and reviewed the manuscript, M.Q, and M.S. assisted with the experiments and reviewed the manuscript, R.C., W.D. and S.S. performed experiments to validate the CLL-MSC co-culture assays in their respective laboratories, W.W., Z.E., M.K., M.S., and U.J. provided patients’ samples and reviewed the manuscript, V.G., N.K and W.P. helped with the design of the study and reviewed the manuscript, and J.B. designed the research, supervised the study, analyzed the data, and revised the paper. The authors state that they have no conflict of interest to declare.
References


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FIGURE LEGENDS

Figure 1. Phenotype of the different MSC
Figure displays phase contrast photomicrographs that depict the morphological appearance of MSC lines and primary MSC derived from the marrow of CLL patient. Cells were imaged in medium using a phase contrast microscope (Model ELWD 0.3, Nikon Inc., Garden City, NY) with a 10×/0.25 NA objective lens. Images were captured with a Nikon D40 digital camera (Nikon Corp., Japan) using Camera Control Pro software (Nikon Corp., Japan); when necessary, Adobe Photoshop 9.0 (Adobe Systems, San Jose, CA) was used for image processing.

Figure 2. Murine MSC protect CLL cells from spontaneous and F-ara-A induced apoptosis
Displayed are the mean relative viabilities of CLL cells in absence or presence of murine MSC and/or 10μM F-ara-A at the time points shown on the horizontal axis. Results represent data for 20:1 CLL-MSC ratios, and are the mean (± SEM) relative viabilities, compared to untreated controls (100%) from 5 different CLL patients for each MSC cell line. All murine MSC cell lines provided significant levels of protection from F-ara-A-induced apoptosis. The names of the murine MSC are displayed above each diagram.

Figure 3. Human MSC protect CLL cells from spontaneous and drug-induced apoptosis
Displayed are the mean relative viabilities of CLL cells in absence or presence of murine MSC and/or 10μM F-ara-A at the time points shown on the horizontal axis. Results represent data for 20:1 CLL-MSC ratios, and are the mean (± SEM) relative viabilities, compared to untreated controls (100%) from 3-5 different CLL patients for each MSC cell line. All human MSC cell lines (except for the primary hMSC pt#2, see Tab. S3) provided significant levels of protection from F-ara-A-induced apoptosis, although the levels of protection generally were lower than those provided by murine MSC. The names of the human MSC are displayed above each diagram.

Figure 4. Co-culture with MSC protects CLL cells from dexamethasone-induced apoptosis
This bar diagram depicts the mean (±SEM) relative viabilities of CLL cells treated with dexamethasone in the presence or absence of murine (KUSA H) or human (StromaNKTer) MSC at the time points displayed on the horizontal axis from 5 different CLL patients. *Significant protection from dexamethasone-induced cytotoxicity compared with control sample (p< .05) (B) Contour plots from a representative CLL sample depict viability of CLL cells, as determined by staining with DiOC₆ and PI, after 48 hours of incubation with 10μM dexamethasone in absence or presence of MSC, as indicated above each of the plots. The percentage of viable cells is displayed above each of the gates that define viable cells (DiOC₆ bright PI exclusion).

Figure 5. 4-HC and combinations of 4-HC and F-ara-A induce high levels of cytotoxicity, even in the presence of MSC

CLL cells were cultured with or without MSC and with 10μM F-ara-A and/or 60μM 4-hydroxycyclophosphamamide. (A) This bar diagram depicts the mean (±SEM) relative viabilities of CLL cells treated with F-ara-A, 4-HC or combination of F-ara-A and 4-HC after 24 hours. Results are presented as mean relative viability compared to untreated controls (100%) and are the mean±SEM viabilities of CLL samples from 8 different patients. *-** indicate significant increases in cytotoxicity of the combination of 4-HC plus F-ara-A when compared to 4-HC alone (p< .05, or p< .01). (B) Contour plots show viability of CLL cells after 24 hours of incubation for one representative patient. The percentage of viable cells (DiOC₆ bright PI exclusion) is shown above each of the gates.

Figure 6. Direct cell-to-cell contact is essential for cell-adhesion mediated drug-resistance

CLL cells were treated with 10 μM F-ara-A and incubated with KUSA H1 (A, upper figure) and StromaNKTer (A, lower figure) in presence or absence of micropore membrane insert for 24, 48 and 72 hours. Bars represent the mean viability of CLL cells compared to untreated control (100%). Data shown are the mean±SEM of six independent experiments. *Significant protection of CLL cells from F-ara-A-induced apoptosis in direct CLL-MSC contact compared with control sample (p< .05) (B) Presented are contour plots of CLL cells from a representative patient after 48 hours of co-culture with StromaNKTer in the conditions indicated above and on the side of the plots. The relative percentages of...
viable cells are displayed above each of the gates. Direct cell-to-cell contact is essential not only for protection from fludarabine-induced apoptosis, but also from spontaneous apoptosis.

**Figure 7. Mcl-1 and PARP expression in CLL cells co-cultured with MSC**

CLL cells were cultured with the MSC lines displayed on the top horizontal axes or in suspension (“Control”) for 48h with or without 10μM F-ara-A (labeled “Ctr” or “+F”, respectively). Then, cleaved and uncleaved Mcl-1 and PARP were analyzed by western blotting, and the respective immunobands are indicated on the left hand side. Cell viability for each condition was measured by flow cytometry, and the percentage of viable cells is displayed below each of the blots. In most cases, MSC co-culture up-regulated Mcl-1 and PARP expression when compared to CLL cells in suspension (control at 48h versus the “Ctr” bands in the presence of MSC). Suspension culture of CLL cells results in spontaneous apoptosis, with associated Mcl-1 and PARP cleavage (second lane from the left in A and B), which was paralleled by a decrease in CLL cell viability from 99% to 47% in A, and from 96% to 81% in B. Treatment with fludarabine resulted in cleavage of the vast majority of Mcl-1 and PARP in the absence of MSC (fifth lane from the left in A, B). This was largely inhibited, sometimes almost abrogated (for example: lanes 6,7 in A) by the presence of MSC. Displayed are western blots of CLL B cell lysates from two representative patients (A, B).
Figure 2

Viability (%)

M210B4

ST-2

KUM4

KUSA H1

Time (h)

+MSC, no F-ara-A

+MSC, +F-ara-A

no MSC, +F-ara-A
Figure 3

Viability (%)

Time (h)

StromaNKTer1

UE6E7T2

UCB408E6E7Ter133

hMSC pt#1

hMSC pt#2

- +MSC, no F-ara-A
- +MSC, +F-ara-A
- no MSC, +F-ara-A
Figure 4

Graph A shows viability (%) over time (h) with different treatments: +Dexamethasone, +Dexamethasone + KUSA H, and +Dexamethasone + Stroma NK Tert.

Graph B illustrates the PI distribution with corresponding percentages: 40.4% for No MSC, 78.8% for +KUSA H1, and 97.7% for +Stroma NK Tert.
Figure 5

A

Viability (%)

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<th>Condition</th>
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<td>+F-ara-A+60μM 4-HC</td>
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B

Flow cytometry images showing different conditions:

- Control
- +10μM F-ara-A
- +60μM 4-HC
- +10μM F-ara-A + 60μM 4-HC

Legend:
- PI
- ΔI/Δ0

Conditions:
- No MSC
- +KUSA H
- +Stroma NKTert
Figure 6

A. Viability (%) of KUSA H cells over time (h) with treatments indicated.

B. Viability (%) of StromaNK1Tert cells over time (h) with treatments indicated.

C. Imaging of cell death markers (PI and DIOC₅) with and without inserts.

Legend:
- +10μM F-ara-A
- +ara-A+MSC
- +F-ara-A+MSC+insert

Control conditions are shown for comparison.
Figure 7

A

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<th>+NKter</th>
<th>+KUSA H</th>
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|   |         |         |     |         |         |       |       |     |         |         |
| PARP |      |        |    |         |         |       |       |     |         |         |
| PARP cleaved | | | | | | | | | |

|   |         |         |     |         |         |       |       |     |         |         |
| Viability, % | 99 | 47 | 96 | 75 | 13 | 99 | 94 | 97 | 73 | 94 |

|   |         |         |     |         |         |       |       |     |         |         |
| Actin |      |        |    |         |         |       |       |     |         |         |

B

<table>
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|   |         |         |     |         |         |       |       |     |         |         |
| PARP |      |        |    |         |         |       |       |     |         |         |
| PARP cleaved | | | | | | | | | |

|   |         |         |     |         |         |       |       |     |         |         |
| Viability, % | 96 | 81 | 98 | 60 | 30 | 99 | 72 | 89 | 59 | 97 |

|   |         |         |     |         |         |       |       |     |         |         |
| Actin |      |        |    |         |         |       |       |     |         |         |
Diverse marrow stromal cells protect CLL cells from spontaneous and drug-induced apoptosis: development of a reliable and reproducible system to assess stromal cell adhesion-mediated drug resistance

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