

Evaluation of Chemosensitivity of Human Bone Marrow Stromal Cells – Differences between Common Chemotherapeutic Drugs

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Abstract. *Background:* Bone marrow stromal cells (BMSCs) are essential for normal hematopoiesis, but also support the growth and survival of malignant hematopoietic cells. There are currently no data available regarding the sensitivity of BMSCs to cytotoxic drugs widely used in the therapy of hematological diseases such as multiple myeloma or acute leukemia. *Materials and Methods:* The chemosensitivity of the BMSC line HS-5 and of primary BMSCs from patients were evaluated in comparison to NCI-H929 myeloma and U937 leukemia cells. *Results:* Both HS-5 cells and human BMSCs showed substantial cell death in response to chemotherapy. While alkylating agents (melphalan, treosulfan) and doxorubicin demonstrated marked BMSC toxicity, nucleotide analogs (gemcitabine, cytarabine) induced only limited BMSC apoptosis. *Conclusion:* Our data suggest that the BMSC toxicity of cytotoxic compounds should be considered in chemotherapeutic regimens.

The bone marrow microenvironment plays a vital role in the homeostasis of the hematopoietic system. Interestingly, malignant hematopoietic cells are also dependent on the support of bone marrow stromal cells (BMSC) (for review see 1, 2). Furthermore, it has been shown that the adhesion of tumor cells to BMSC induces strong drug resistance (3, 4). Despite this central role of BMSC in the regulation of healthy and malignant hematopoietic cell growth and survival, there are currently no data available regarding the sensitivity of BMSCs to chemotherapeutic drugs. The

present study systematically evaluates the chemosensitivity of BMSCs in comparison to malignant myeloma and leukemia cells.

Materials and Methods

Cells. The NCI-H929, U937 and HS-5 cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA), grown in RPMI 1640 medium (Boehringer, Ingelheim, Germany) containing 20% heat-inactivated fetal calf serum (Boehringer) in a humidified atmosphere (37.5 °C; 5% CO₂), and seeded at a density of 1x10⁵ cells/ml. After informed consent, mononuclear cells from bone marrow aspirates were grown in plastic flasks to a confluent, adherent monolayer, as previously described (4). The ethical committee of the University of Munich, Germany, approved the study.

Reagents. Cytarabine and gemcitabine were purchased from Sigma-Aldrich (Seelze, Germany), melphalan from GlaxoSmithKline (Munich, Germany), treosulfan from Medac (Wedel, Germany), and doxorubicin from Pharmacia (Erlangen, Germany).

Analysis of apoptosis by flow cytometry. After 2 washes, 100 µl of the cell suspension were incubated with 10 µl of 50 µg/ml propidium iodide (PI) for 15 min at room temperature in the dark. The cells were analyzed by flow cytometry (Coulter EPICS XL-MCLTM; System IITM) within 30 min.

Cytotoxicity assay. For the quantification of viable cells, a WST-1 viability assay protocol was used, as recommended by the manufacturer (Roche, Penzberg, Germany). The cells were transferred to new wells of a microtiter plate and the absorbance at 440 nm was measured using a microplate ELISA reader to detect metabolically intact cells (reference wavelength: 680 nm). The proportion of cell death was calculated in comparison to untreated controls.

Statistics. The mean values with standard deviations from representative experiments are shown in the figures. The Kruskal-Wallis one-way analysis of variance on ranks was used to determine the statistical significance of treatment results. The pairwise multiple comparison procedure was performed according to Dunn's method. Values of *p*<0.05 were considered statistically significant.

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Results

The human bone marrow stromal cell line HS-5 was incubated with increasing concentrations of melphalan, treosulfan, doxorubicin, gemcitabine and cytarabine for two days, as shown in Figure 1A. The used concentrations are within the ranges commonly used *in vitro* and are achievable in humans. Maximal serum concentrations are up to 1.6 mM treosulfan (standard dose therapy) and 4.5 mM treosulfan (high-dose therapy) (5, 6), 170 µM gemcitabine (data provided by the manufacturer), 0.6 µM cytarabine (standard dose therapy) (data provided by the manufacturer), 1.67 µM doxorubicin (7) and 150 µM melphalan (8). All these compounds are effective in the therapy of acute myeloid leukemia or multiple myeloma (9, 10). We selected the multiple myeloma cell line NCI-H929 for reference. As cytarabine is uncommon in the therapy of multiple myeloma, the acute myeloid leukemia cell line U937 served as reference for this compound. Cell death was determined by propidium iodide (PI)-uptake using flow cytometry. As seen in Figure 1A, the alkylating agents melphalan and treosulfan, as well as the nucleotide analogs gemcitabine and cytarabine, induced considerable cell death in the HS-5 stromal cell line without reaching a plateau, although this effect was significantly inferior compared to that for the NCI-H929 or U937 cells. Of note, except for doxorubicin, concentrations that led to more than 50% cell death in the stromal cell line corresponded to clinically achievable plasma levels in patients.

Since HS-5 are rapidly proliferating, immortalized cells, the clinical relevance of the above data was verified with a second model with primary human BMSCs. These hBMSCs were from 8 bone marrow aspirates of consecutive patients with diagnoses of either multiple myeloma or acute leukemia (Table I). Long-term bone marrow cultures were grown until a confluent monolayer was achieved. As these very large and adherent cells cannot be analyzed by flow cytometry, the WST-1 cytotoxicity assay was used to detect metabolically intact cells. The ratios in Figure 1B representing cell viability were calculated from the absorbance of the treated *versus* the untreated samples. Five out of the 8 patients received previous chemotherapy, 3 were at diagnosis. All patients had a malignant hematological disease. Our experiments confirmed that all 5 drugs impaired the viability of hBMSC. Whereas the alkylating agents melphalan and treosulfan led to a marked and dose-dependent reduction in cell viability of hBMSCs, nearly reaching the values of the myeloma reference. The doxorubicin treatment resulted in a 50% reduction of viability with a plateau at the used concentrations. In contrast, comparably little effect was observed using gemcitabine and cytarabine. Interestingly, we saw a substantial inter-patient variability of hBMSC viability that did not correlate with diagnosis or prior treatment.

Discussion

Preclinical chemosensitivity testing using human cell lines and malignant cells obtained from patients has commonly been used in hematology and oncology to evaluate the efficacy of new cytotoxic compounds and drug combinations for several decades. The proliferation, cell death, differentiation, migration and mechanisms of action can be easily studied *in vitro*. More recently, it has been shown that the bone marrow microenvironment is essential in the pathogenesis of hematological diseases like multiple myeloma or leukemia. Therefore, *in vitro* models of bone marrow microenvironments, such as cytokine substitution, fibronectin-coated plates and, most recently, BMSC monolayers, have been developed and used for the evaluation of new cytotoxic compounds such as bortezomib or revlimid. Although the influence of BMSCs on the chemosensitivity of malignant cells has been intensively examined in these studies, no data are available regarding the sensitivity of BMSC itself to cytotoxic drugs.

Our current *in vitro* experiments demonstrate a considerable susceptibility of hBMSCs to cytotoxic drugs. BMSC cell death was induced at commonly used dose levels, emphasising the relevance of the study. There are certain differences between the results of the HS-5 cell line and the primary stromal cells, most probably due to the completely different growth pattern. Knowledge about the chemosensitivity of the rapidly proliferating HS-5 cell line is important for the design of upcoming preclinical studies using co-cultures of myeloma/leukemia cells and BMSC. The data regarding the primary stromal cells from patients show striking differences between the cytostatic drugs employed. The alkylating agents seemed to induce a more pronounced BMSC toxicity in comparison to that induced by the nucleotide analogs. Interestingly, recent studies have shown that integrin binding of malignant and stromal cells mediates primary drug resistance in various hematological neoplasias and solid tumors (3, 11). As the abrogation of integrin binding reduces the protective effect of this interaction (4, 12), a decrease of primary drug resistance and, at least in part, enhancement of tumor cell apoptosis should be expected by impairing stromal cell viability. Conversely, conditioning regimens with strong BMSC toxicity could impair engraftment after stem cell transplantation. Additionally, while considerable knowledge has accumulated regarding the long-term toxicity of cytotoxic drugs upon hematopoietic precursor cells, *e.g.* VP-16-induced acute leukemias, little is known about the long-term toxicity of these compounds on stromal cells.

In light of the data obtained, additional research is necessary to translate these preclinical data into clinical trials. Furthermore, the role of BMSC toxicity in drug-induced myelosuppression, rejection of stem cell transplants, and cell

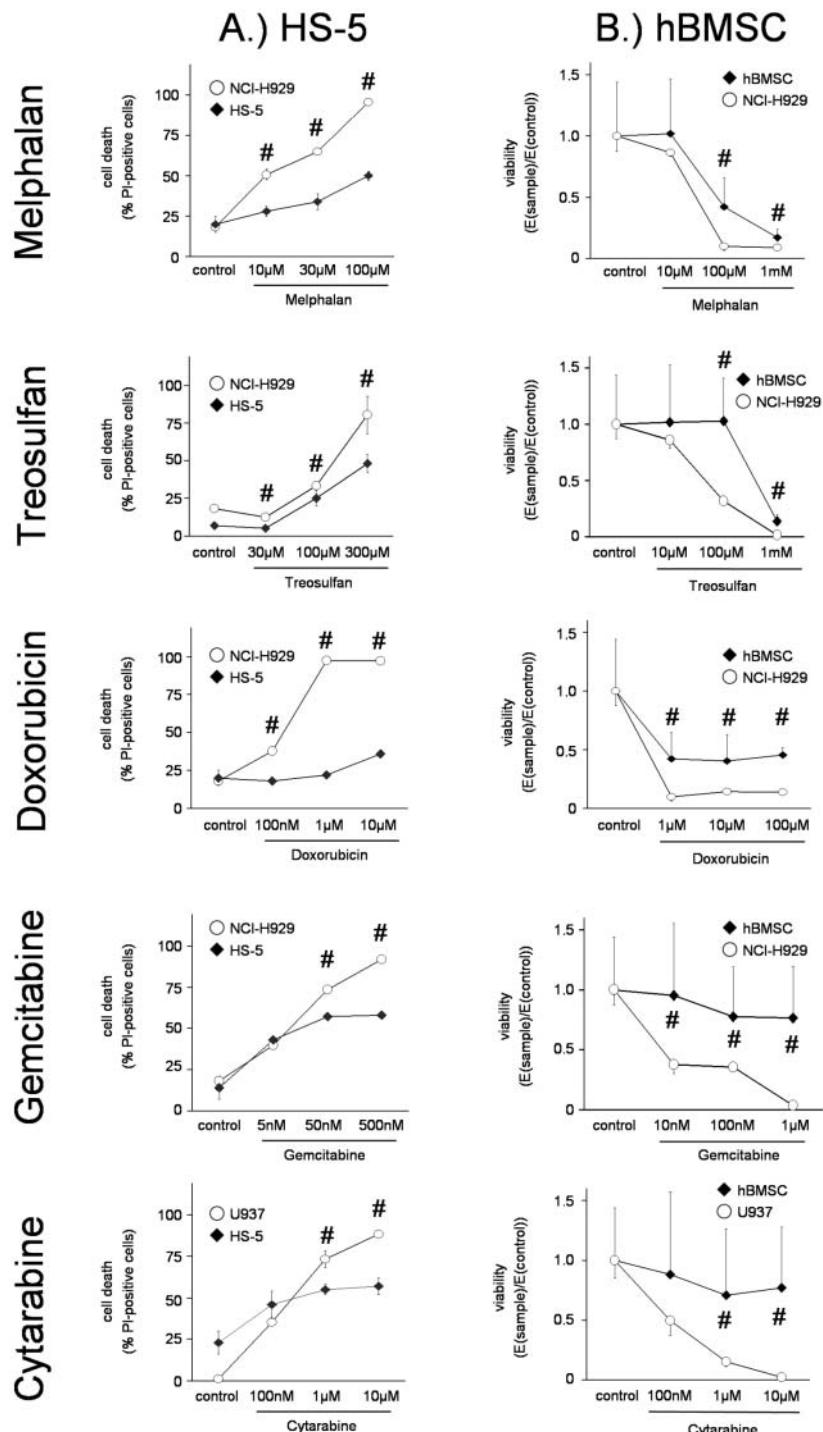


Figure 1. Chemosensitivity of HS-5 cells and human bone marrow stromal cells (hBMSC). (A) The HS-5 cells were treated with increasing concentrations of melphalan, treosulfan, doxorubicin, gemcitabine and cytarabine for 48 h as indicated. Cell death was determined by uptake of propidium iodide (PI) by flow cytometry. The NCI-H929 and U937 cells served as controls. The mean values of at least 2 independent experiments and standard deviations are shown. Statistics of HS-5 cells in comparison to NCI-H929/U937 were performed. P-values <0.05 are indicated by '#'. (B) The human bone marrow stromal cell (hBMSC) long-term cultures were grown from bone marrow aspirates of 8 consecutive patients. The confluent monolayers were treated for 48 h with increasing concentrations of melphalan, treosulfan, doxorubicin, gemcitabine and cytarabine as indicated. The NCI-H929 and U937 cells served as controls. Cell viability was determined by the WST-1 assay. Absorbance by formazan dye indicates metabolically active cells. Cell viability is shown as the ratio of absorbance of sample cells and the absorbance of untreated control cells (mean values and standard deviations). Statistics of hBMSCs in comparison to NCI-H929/U937 were performed. P-values <0.05 are indicated by '#'.

Table I. Patient characteristics.

Patient	Age	Sex	Diagnosis	Prior treatment
1	75	f	Multiple myeloma (IgG-kappa; III-B)	3xVAD, 1xEC, 2xHD-Mel
2	77	f	MGUS	No treatment
3	51	f	Multiple myeloma (asecretory, II-A)	4xID, 1xIEV, 2xHD-Mel
4	29	f	ALL	Induction I/II of GMALL-protocol
5	51	m	Multiple myeloma (lambda-LC, III-B)	6xVAD
6	50	m	Multiple myeloma (IgG-kappa, III-A)	At diagnosis
7	51	m	Multiple myeloma (lambda-LC, III-B)	6xVAD
8	51	m	Multiple myeloma (IgD-lambda, III-A)	At diagnosis

VAD: Vincristine, Doxorubicin, Dexamethasone

EC: Etoposide, Cyclophosphamide

HD-Mel: High-dose Melphalan with autologous stem cell support

GMALL: Methotrexate, Cyclophosphamide, Dexamethasone, Vincristine, Daunorubicin, PEG-Asparaginase, Cytarabine, 6-Mercaptopurine

LC light chain

MGUS monoclonal gammopathy of uncertain significance

ALL acute lymphoblastic leukemia

adhesion-mediated drug resistance needs to be evaluated. Therefore, new chemotherapeutic drugs and regimens should be characterized regarding their BMSC toxicity.

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References

- Dalton WS: The tumor microenvironment: focus on myeloma. *Cancer Treat Rev* 29 Suppl 1: 11-9, 2003.
- Hall BM and Gibson LF: Regulation of lymphoid and myeloid leukemic cell survival: role of stromal cell adhesion molecules. *Leuk Lymphoma* 45: 35-48, 2004.
- Hazlehurst LA, Landowski TH and Dalton WS: Role of the tumor microenvironment in mediating *de novo* resistance to drugs and physiological mediators of cell death. *Oncogene* 22: 7396-7402, 2003.
- Schmidmaier R, Baumann P, Simsek M, Dayyani F, Emmerich B and Meinhardt G: The HMG-CoA reductase inhibitor simvastatin overcomes cell adhesion mediated drug resistance (CAM-DR) in multiple myeloma by geranylgeranylation of Rho protein and activation of Rho kinase. *Blood* 104: 1825-1832, 2004.
- Hilger RA, Harstrick A, Eberhardt W, Oberhoff C, Skorzec M, Baumgart J, Seeber S and Scheulen ME: Clinical pharmacokinetics of intravenous treosulfan in patients with advanced solid tumors. *Cancer Chemother Pharmacol* 42: 99-104, 1998.
- Scheulen ME, Hilger RA, Oberhoff C, Casper J, Freund M, Josten KM, Bornhauser M, Ehninger G, Berdel WE, Baumgart J, Harstrick A, Bojko P, Wolf HH, Schindler AE and Seeber S: Clinical phase I dose escalation and pharmacokinetic study of high-dose chemotherapy with treosulfan and autologous peripheral blood stem cell transplantation in patients with advanced malignancies. *Clin Cancer Res* 6: 4209-4216, 2000.
- Swenson CE, Bolcsak LE, Batist G, Guthrie TH Jr, Tkaczuk KH, Boxenbaum H, Welles L, Chow SC, Bhamra R and Chaikin P: Pharmacokinetics of doxorubicin administered i.v. as Myocet (TLC D-99; liposome-encapsulated doxorubicin citrate) compared with conventional doxorubicin when given in combination with cyclophosphamide in patients with metastatic breast cancer. *Anti-cancer Drugs* 14: 239-246, 2003.
- Reece PA, Hill HS, Green RM, Morris RG, Dale BM, Kotasek D and Sage RE: Renal clearance and protein binding of melphalan in patients with cancer. *Cancer Chemother Pharmacol* 22: 348-352, 1988.
- Meinhardt G, Dayyani F, Jahrsdorfer B, Baumgart J, Emmerich B and Schmidmaier R: Treosulfan is an effective inducer of cell death in myeloma cell lines and primary myeloma cells from patients. *Br J Haematol* 122: 892-899, 2003.
- Schmidmaier R, Oellerich M, Baumgart J, Emmerich B and Meinhardt G: Treosulfan-induced apoptosis in acute myeloid leukemia cells is accompanied by translocation of protein kinase C delta and enhanced by bryostatin-1. *Exper Haematol* 32: 76-86, 2004.
- Damiano JS, Hazlehurst LA and Dalton WS: Cell adhesion-mediated drug resistance (CAM-DR) protects the K562 chronic myelogenous leukemia cell line from apoptosis induced by BCR/ABL inhibition, cytotoxic drugs, and gamma-irradiation. *Leukemia* 15: 1232-1239, 2001.
- Damiano JS, Cress AE, Hazlehurst LA, Shtil AA and Dalton WS: Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. *Blood* 93: 1658-1667, 1999.

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