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Bone marrow stromal cells attenuate mitoxantrone cytotoxicity against HL-60 leukemic cells

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We evaluated the effect of cultured human bone marrow (BM) stromal cells on the cytotoxicity of mitoxantrone (MIT) against the HL-60 leukemic cell line. BM-derived fibroblastoid cells (BMFC) suppressed colony formation of HL-60 cells in the absence of MIT. BMFC increased the survival of HL-60 colony-forming cells in the presence of 50 ng/ml MIT. A significant decrease in the drug concentration was not detected on BMFC. Our results suggest that BM stromal cells can modulate the antileukemic effect of an anticancer drug.

Key words: Bone marrow stromal cells, HL-60 leukemia cells, mitoxantrone.

Introduction

Relapse of acute leukemia has been ascribed to the existence of drug-resistant populations among untreated leukemic cells.[1] However, the proliferating leukemic cells at relapse can often be eradicated with the drugs previously administered during the remission induction, which suggests that mechanism(s) other than drug resistance were protecting the leukemic cells from the chemotherapeutic agents.

Bone marrow (BM) is the most prevalent site for leukemia relapse. Hematopoietic stem cells are maintained in close proximity to BM stromal cells.[2] One attractive possibility is that leukemic stem cells are closely associated with the BM stromal cells. Therefore, we studied the effect of cultured BM stromal cells on the cytotoxicity of an antileukemic agent, mitoxantrone (MIT),[3] against an acute myeloid leukemia cell line, HL-60.[4]

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Materials and methods

Preparation of BM-derived adherent cells

BM aspirates were obtained from acute leukemia patients in complete hematological remission. A BM mononuclear cell fraction, isolated by centrifugation over Percoll (1.085 g/ml), was placed in a 24-well collagen-coated dish (Adcellcoat; Toyobo) at 1.0×10^6 cells/well in 1.0 ml RPMI 1640 supplemented with 10% pooled heat-inactivated human AB group serum (HI-ABS) (hereafter referred to as ABS-RPMI). Medium conditioned by phytohemagglutinin-stimulated peripheral blood mononuclear cells (PHA-LCM) was prepared by culturing 1.0×10^6 cells/ml for 1 week with 0.2% PHA-P. PHA-LCM at 30% was supplemented to culture BM macrophage-like cells (BMMϕ). Methylprednisolone (Solu-Medrol; Upjohn) (2 μM) was added to culture BM fibroblastoid cells (BMFC).[5] All cultures were maintained at 37°C in humidified 5% CO_2 in air, with weekly total medium replacement. Confluent monolayers of BM-derived adherent cells composed of BMMϕ or BMFC were seen within 3 weeks of culture.

Cell line

HL-60, confirmed to be free from mycoplasma infection, was kindly provided by K Takada (Nihon Kayaku Co., Ltd), maintained in RPMI 1640 with 5% HI-ABS and passed twice weekly. Viability was assessed by trypan blue dye exclusion. Type I collagen, with which the 24-well dish (Adcellcoat)

is coated, has been reported not to induce differentiation of HL-60 cells.[6]

Binding assay

The binding of HL-60 cells to the BM adherent cell layers was assessed by the method of Somers *et al.*[7] with minor modifications. In brief, 1.0×10^6 viable HL-60 cells suspended in 1.0 ml ABS-RPMI were incubated with 50 μCi of ^{111}In oxine for 15 min at room temperature. The labeled HL-60 cells were washed three times, resuspended at 1.0×10^5 cells/ml in ABS-RPMI, and 1.0 ml of the HL-60 cell suspension was layered onto each BM adherent cell layer. After 60 and 120 min of incubation at 37°C, non-adherent HL-60 cells were removed by washing vigorously twice with Hank's balanced salt solution (HBSS). The remaining adherent cells were lysed with 1.0 ml of 1.0% sodium dodecyl sulfate. After 10 min at room temperature, a 0.1 ml aliquot of the content of each well was counted in an automatic gamma spectrometer (ANSR, Abott Laboratories). The percentage binding was calculated by dividing by the count of 0.1 ml labeled HL-60 cells.

Co-culture with BM adherent cells and colony assay

Logarithmically growing HL-60 cells at 1.0×10^5 viable cells/ml in 1.0 ml ABS-RPMI were layered onto the BM adherent cell layers which had been washed twice with ABS-RPMI. After 120 min of incubation at 37°C, non-adherent HL-60 cells were collected as described above. For colony assay, 1.0×10^4 harvested cells were plated on a 35 mm culture dish (Falcon 3001) in 1.0 ml Iscove's modified Dulbecco's medium containing 10% HI-ABS, 10% PHA-LCM and 0.3% methylcellulose. Colonies of more than 20 cells were scored after 14 days of incubation at 37°C in 5% CO_2. The number of HL-60 colonies was proportional to the number of viable HL-60 cells in the range 1.0×10^3 to 1.0×10^4 cells/ml.

Drug sensitivity assay (Figure 1)

MIT (Novantron; kindly provided by Lederle Japan Ltd) was present at 50 ng/ml during the last 60 min of the co-culture. HL-60 cells were harvested as described above. All of the HL-60 cells recovered from a well were seeded into a dish for colony assay. The surviving fraction was calculated by dividing by the number of HL-60 colonies after MIT-free co-culture on the same kind of BM adherent cells.

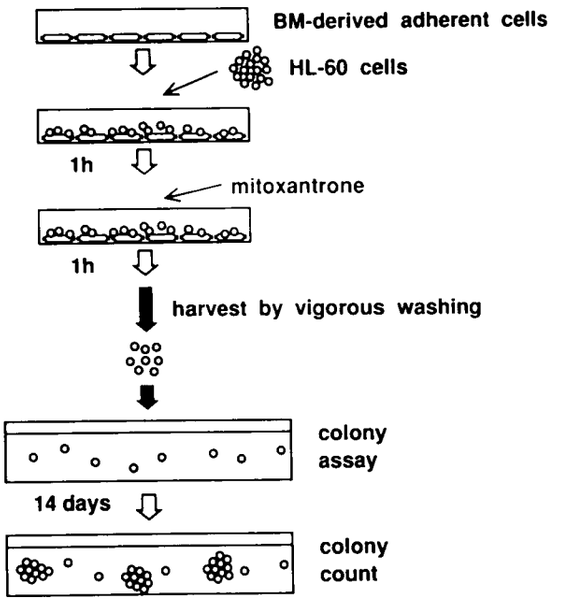

Figure 1. Assay of the effect of BM adherent cells on the cytotoxicity of MIT against HL-60 colony-forming cells.

In accordance with the manufacturer's package insert, after administration of MIT at the clinically recommended dose of 10 mg/m^2 to patients, a peak plasma level of 553 ng/ml was attained, and the concentration decreased to 19 ng/ml in 120 min. The concentration of MIT (50 ng/ml) employed in this study is comparable with that obtained in clinical settings. A recent study has indicated that MIT does not induce differentiation of HL-60 cells.[8]

Measurement of drug concentration

ABS-RPMI containing 50 ng/ml of MIT was layered onto the dishes with or without BMFC and incubated at 37°C for 15 min. The medium was collected, centrifuged to remove cells and stored at −20°C. The concentration of MIT was measured by high pressure liquid chromatography.

Results and discussion

To assess the harvest of HL-60 cells after co-culture, a binding assay was performed using radiolabeled

Table 1. Binding of HL-60 cells to BM-derived adherent cells

BM adherent cells	Binding (%)[a]	
	60 min	120 min
—	0	0.2 ± 0.1
BMMϕ	1.7 ± 0.4	1.8 ± 0.4
BMFC	14.7 ± 0.6	25.8 ± 0.9

[a] Results are expressed as the means ± SE of three experiments with BM adherent cells obtained from three different patients.

HL-60 cells. As shown in Table 1, 26% of seeded HL-60 cells remained bound to BMFC and could not be recovered by vigorous washing. In contrast, more than 98% could be recovered from BMMϕ or collagen-coated dishes without BM adherent cells.

Of the HL-60 cells seeded onto BMFC, 74% were harvested after 120 min of co-culture. As shown in Table 2, colony formation by the HL-60 cells harvested from BMFC was significantly diminished, indicating that BMFC suppressed the clonogenicity of HL-60 cells during the 120 min of co-culture. In contrast, BMMϕ did not significantly affect colony formation. Although it has been reported that BM fibroblasts have no effect on the colony number of HL-60,[9] BM fibroblasts and HL-60 cells were separated by agar in that experiment. Several studies have suggested that BM stromal cells inhibit proliferation[10,11] and induce differentiation[12] of HL-60 cells via direct cell-to-cell contact. Extracellular matrix components, which are closely associated with BM stromal cells, have been reported to inhibit proliferation and to induce maturation of HL-60 cells.[6,13]

The surviving fraction of HL-60 clonogenic cells increased 3-fold when they were treated with MIT on BMFC (Table 2). Because MIT did not decrease the binding of HL-60 cells to BMFC (data not shown), the artifactual increase in surviving colonies due to an increased harvest from BMFC was excluded. The number of surviving colonies per seeded HL-60 cell increased approximately 2.2-fold. That excludes the possibility that a MIT-sensitive subpopulation of HL-60 cells bound to BMFC and their depletion caused an apparent increase in the surviving fraction of harvested cells. In that case, an absolute increase in the number of surviving colonies per seeded HL-60 cell would not be expected. Therefore our findings indicate that BMFC can rescue some of the HL-60 colony-forming cells from the cytotoxicity of MIT. In contrast, BMMϕ had no significant influence on HL-60 survival (Table 2).

The concentration of MIT in the medium was measured to assess the degradation of MIT by BMFC. After 15 min of incubation in the absence or presence of BMFC, 23.4 ± 2.5 and 20.4 ± 4.3 ng/ml (mean ± SE, $n = 3$) was detected, respectively (not statistically different with the Wilcoxon signed-rank test).

MIT has a cytocidal effect on both proliferating and non-proliferating cells. However, stationary (G_0) phase cells are significantly more resistant to the cytotoxic action than cycling cells.[15] The colony formation or MIT sensitivity of an acute monoblastic leukemia cell line, YK-M2,[14] was not affected by BMFC (data not shown). Although the possibility of a significant decrease in drug concentration in the vicinity of BMFC cannot be completely excluded, we consider it more likely that BMFC protected the HL-60 colony-forming cells by inhibiting colony growth.

We found that cultured BM stromal cells can protect an acute myeloid leukemia cell line, HL-60, from a chemotherapeutic agent, MIT. Evidence has been accumulating that tumor cells treated with

Table 2. Effect of BM-derived adherent cells on HL-60 colony formation and on cytotoxicity of MIT against HL-60 colony-forming cells[a]

BM adherent cells	Colony/10^3 HL-60 cells[b]		Surviving fraction (%)[c]	
—	125.7 ± 14.4	NS[d]	2.7 ± 1.1	NS[d]
BMMϕ	127.7 ± 23.3		3.1 ± 1.2	
BMFC	73.8 ± 10.8	$p < 0.03$[d]	8.1 ± 2.3	$p < 0.03$[d]

[a] BM aspirates were obtained on six occasions from four different patients. Results are displayed as mean ± SE, $n = 6$.
[b] The number of colonies divided by the number of harvested HL-60 cells.
[c] Surviving fraction of HL-60 colony-forming cells after treatment with 50 ng/ml MIT for 60 min.
[d] Statistical analysis was performed with the Wilcoxon signed-rank test. NS: not statistically different.

anti-cancer drugs die through apoptosis[16] and BM stromal cells have been reported to protect B lineage acute lymphoblastic leukemia cells from apoptosis.[17] Modulation of BM stromal cells might be another approach to improve the antileukemic effect of chemotherapeutic agents

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