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SHORT COMMUNICATION

Britt Sundman-Engberg · Christer Paul · Ulf Tidefelt

Effect of cytokines on the toxicity of cytostatic drugs to normal bone marrow cells in vitro

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Abstract We evaluated in vitro how growth factors influenced the effect of cytostatic drugs on normal hematopoietic progenitor cells. Bone marrow was obtained from 15 donors for bone marrow transplantation. After separation the mononuclear fraction was incubated with granulocyte colony-stimulating factor (G-CSF) at 5 and 50 ng/ml, with granulocyte/macrophage colony-stimulating factor (GM-CSF) at 1 and 10 ng/ml, and with interleukin 3 (IL-3) at 0.5 and 5 ng/ml for 24 h prior to incubation with cytostatic drugs. These incubations were performed with 0.05 μM mitoxantrone and 0.2 μM daunorubicin for 1 h, and cells were thereafter cultured for colony-forming units – granulocyte/macrophage (CFU-GM) in soft agar for 10–12 days. Incubation with 0.05 μM cytosine arabinoside was performed continuously throughout the culture period. The proliferation of normal hematopoietic progenitor cells stimulated with GM-CSF at 10 ng/ml and with IL-3 at 5 ng/ml was significantly increased to 218% and 215% colonies, respectively, as compared with the control stimulated with conditioned medium only. Stimulation with G-CSF, on the other hand, did not induce any significantly enhanced proliferation relative to the control. Daunorubicin applied in combination with G-CSF at 5 ng/ml or with IL-3 at 0.5 ng/ml exerted a significantly higher degree of cytotoxicity on normal hematopoietic progenitor cells, resulting in 21% and 30% surviving colonies as compared with the 38% recorded for daunorubicin alone ($P < 0.05$). Neither G-CSF nor IL-3 at a higher concentration nor GM-CSF exerted a significantly altered degree of toxicity relative to cells incubated with daunorubicin alone. The cytotoxic effect exerted on normal hematopoietic cells by mitoxantrone or ara-C was unchanged or significantly decreased after stimulation with growth factors as compared with the effect on cells incubated with cytostatic drugs alone. We conclude that G-CSF and IL-3 augment the effect of daunorubicin on normal hematopoietic progenitor cells.

Key words Bone marrow · Normal hematopoietic cells · CFU-GM · Anthracyclines · Ara-C · IL-3 · G-CSF · GM-CSF

Abbreviations *Ara-C* Cytosine arabinoside · *CFU-GM* colony-forming unit – granulocyte macrophage · *DNR* Daunorubicin · *G-CSF* Granulocyte colony-stimulating factor · *GM-CSF* Granulocyte macrophage colony-stimulating factor · *IL-3* Interleukin 3 · *CM* Conditioned medium · *MIT* Mitoxantrone

Introduction

Major reasons for treatment failure in cancer chemotherapy are high levels of toxicity to normal hematopoietic progenitor cells and drug resistance in the malignant cells. In treatment with cytostatic drugs the toxicity to normal hematopoietic progenitor cells is generally the dose-limiting factor. In the clinical situation there are great interindividual differences with regard to bone marrow toxicity. Much of the development of new treatment schedules has the objective of reducing bone marrow toxicity and, thereby, increasing the therapeutic index of anticancer drugs.

Cytotoxic drugs mostly exert their effects on the synthesis of DNA, RNA, or protein, e.g., on cells in the active cell cycle. The anthracycline antibiotic agent daunorubicin and the anthracenedion derivative

B. Sundman-Engberg · C. Paul
Department of Medicine,
Karolinska Institute at Huddinge Hospital,
S-141 86 Huddinge, Sweden

U. Tidefelt
Department of Medicine, Örebro Medical Center Hospital,
S-701 85 Örebro, Sweden

B. Sundman-Engberg (✉)
Division of Clinical Hematology, Huddinge Hospital,
S-141 86 Huddinge, Sweden
Tel.: +46-8-585-83862; Fax: +46-8-585-83810;
e-mail: britt.sundman-engberg@hematol.hs.sll.se

mitoxantrone are thought to be intercalating agents that also form complexes with topoisomerase II and DNA [7, 26]. Cytosine arabinoside (ara-C) is regarded to be more S-phase-specific than the anthraquinones and is active after its phosphorylation to ara-CTP [12, 13]. All dividing cells, including hematopoietic cells, are more vulnerable to these drugs than are cells in the G_0 phase which can be expected to be less sensitive to the effect of ara-C and to that of the anthraquinones.

Growth factors are used to enhance bone marrow proliferation after intensive chemotherapy. The DNA synthesis rate of acute myeloblastic leukemia (AML) cells is frequently low [17]. There is evidence that growth factors may influence the sensitivity of leukemic progenitor cells to cytotoxic chemotherapy by recruiting G_0 cells into the cell cycle [6, 14, 15]. An attractive approach to increase the cytotoxic effect on the tumour cells as well as to enhance the growth of normal bone marrow cells would be to use a combination of growth factors and cytostatic drugs. Reports are available on several clinical studies using growth factors in combination with cytostatic drug treatment, but there is no conclusive evidence as to how the treatment outcome is affected [2, 8–10, 22]. Apparently there is a risk that growth factors could also make normal hematopoietic progenitor cells more sensitive to cytostatic drugs, and this problem has not been extensively addressed.

The main objective of this study was therefore to evaluate in vitro how a combination of growth factors and cytostatic drugs would affect bone marrow cells obtained from healthy donors. The incubation concentrations and times for the drugs were chosen to reach the same intracellular concentrations achieved after in vivo infusion with these drugs [23, 25]. The in vitro intracellular concentrations of daunorubicin and doxorubicin mimicked the intracellular concentrations reached in vivo after infusion of 60 mg/m^2 for 1 h; the corresponding in vivo concentrations were 12 mg/m^2 for mitoxantrone as infused for 1 h and 100 mg/m^2 for ara-C given as a continuous infusion.

Materials and methods

Materials

Cells

Cells were obtained from 15 healthy donors (7–67 years) for bone marrow transplantation. Bone marrow samples of 5–10 ml were collected in preservative-free heparin, and the mononuclear cells were separated on sodium metrizoate/Ficoll (Lymphoprep, Nyegaard & Co, Oslo) [5] and thereafter washed twice with culture medium (RPMI 1640).

Cytostatic drugs

The anthracycline derivative daunorubicin (Cerubidin) was purchased from Rhône-Poulenc Rorer. Stock solutions of 100 μM were stored at −20 °C. Mitoxantrone (Novantrone) was purchased from Lederle/Cyanamid and was freshly prepared before every incubation. Ara-C (Cytosar) was purchased from Upjohn. Stock solutions of 100 μM were made every week and stored at 4 °C.

Growth factors

GM-CSF (granulocyte/macrophage colony-stimulating factor) was purchased from Sandoz as Leucomax. Stock solutions of 1500 ng/ml were stored at −70 °C. The final concentrations used in cultures were 1.0 and 10 ng/ml. G-CSF (granulocyte colony-stimulating factor) was purchased from Roche as Neupogen. Stock solutions of 3000 ng/ml were stored at 4 °C. The final concentrations used were 5.0 and 50 ng/ml. IL-3 (interleukin 3) was purchased from Genzyme as RH-IL-3. Stock solutions of 2000 ng/ml were stored at −70 °C. The final concentrations were 0.5 and 5 ng/ml. Working solutions of growth factors were freshly prepared for every test performed.

Experimental design

Incubation and cultures

In all, 5×10^5 mononuclear cells/ml were incubated for 24 h with phosphate-buffered saline (PBS; control), G-CSF, GM-CSF, or IL-3 at two different concentrations. Incubations were performed in polypropylene tubes with RPMI 1640, 10% fetal calf serum (FCS), and 1% L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2. The final volume was 1 ml, including the growth factors, which were added at 10 times the final concentration.

After 23 h of incubation with growth factors, cytostatic drugs were added for 1 hour without prior washing. Mitoxantrone was added to a final concentration of 0.05 μM and daunorubicin was added to a final concentration of 0.2 μM. Ara-C was first added to a final concentration of 0.05 μM for 1 h but was also installed at the same concentration in the agar cultures as a continuous incubation. The incubation concentrations were chosen according to what we have previously shown to give an intracellular exposure mimicking the clinical situation after pharmacokinetics studies [23, 25].

After the complete incubation period of 24 h the cells were centrifuged (400 *g* for 10 min) and the supernatant was discarded prior to culturing in agar. The agar cultures were done in 35-mm petri dishes with 1 ml in the feeder layer and 1 ml in the over-layer [16]. The feeder layer consisted of 40% double-concentrated McCoy's medium including 30% FCS, 10% CM (conditioned medium) [29], and 50% of 1.0% agar, which gives a final concentration of McCoy's with 15% FCS and 0.5% agar. The final concentration of the over-layer was McCoy's with 15% FCS, 0.3% agar, and 1×10^5 cells/ml. Without a washing step the cells were resuspended in 5 ml agar-medium, of which 1 ml was added to each of three culture dishes. Growth factors were added to the overlayer in the same concentrations described above for the rest of the culture period. For cells incubated with ara-C, fresh ara-C was added at the same concentration used during the culture period. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2, and colonies with more than 40 cells were counted in an inverted microscope after 10–12 days.

Evaluation of cytotoxic effect

For cytostatic drugs alone the toxic effect was expressed as the percentage of colonies counted relative to a drug-free control stimulated with only CM. The combined effect of cytostatic drugs and growth factors was expressed as the percentage of colonies counted relative to a control incubated with the corresponding growth factor.

Statistical analysis

Wilcoxon's signed-rank test was used to evaluate differences between mean values recorded for sensitivity per group of patients. A P value of <0.05 was regarded as statistically significant.

Results

Growth of controls

A mean of 98 bone marrow colonies were detected in 16 controls cultured with CM in the absence of growth factors or cytostatic drugs, with the minimum being 32 and the maximum, 215 colonies/1×10^5 mononuclear cells.

Growth after addition of growth factors

Cells stimulated with G-CSF at 5 or 50 ng/ml did not show a significant increase in proliferation relative to the controls cultured with CM, as is shown in Table 1. The number of colonies increased 1.5 times on incubation with GM-CSF at 1 ng/ml and with IL-3 at 0.5 ng/ml and showed a significant 2-fold increase on incubation with GM-CSF at 10 ng/ml and with IL-3 at 5 ng/ml (to 218% and 216%, respectively; $P < 0.005$) as compared with the control.

Table 1 Stimulating effect of growth factors on normal bone marrow cells from healthy donors. The control value is expressed as the number of colonies/10^5 cells, and the growth-factor-stimulated samples are expressed as percentages of colonies relative to the control stimulated with conditioned medium

		n	Mean unit	Range
Control (CM)		16	98 colonies	32–215
G-CSF	5.0 ng/ml	13	103%	53–216
G-CSF	50 ng/ml	16	113%	65–248
GM-CSF	1.0 ng/ml	13	128%*	80–242
GM-CSF	10 ng/ml	15	218%**	108–391
IL-3	0.5 ng/ml	13	144%**	106–221
IL-3	5.0 ng/ml	15	216%**	105–579

*$P < 0.05$; **$P < 0.005$

Growth after incubation with cytostatic drugs

Incubation for 1 h with 0.2 μM daunorubicin exerted a mean cytotoxic effect that resulted in 38% colonies and 0.05 μM mitoxantrone yielded 20% colonies relative to controls stimulated with CM (Table 2). Continuous incubation with ara-C at a concentration of 0.05 μM gave 18% colonies relative to a control stimulated with CM.

G-CSF and cytostatic drugs

The cytotoxic effect on normal hematopoietic progenitor cells incubated with G-CSF at 5 ng/ml in combination with daunorubicin was significantly increased to 17% as compared with 38% of the colonies ($P < 0.05$) in samples incubated with daunorubicin alone, as is shown in Fig. 1 and Table 2. Incubation with G-CSF at 5 ng/ml also induced a greater effect of mitoxantrone, although this difference was not significant. The addition of G-CSF at 50 ng/ml did not alter the toxicity of daunorubicin or mitoxantrone as compared with treatment with the cytostatic drugs alone. Incubations with either concentration of G-CSF did not significantly change the toxicity of ara-C to normal hematopoietic progenitor cells.

GM-CSF and cytostatic drugs

The cytotoxic effects of daunorubicin or mitoxantrone in combination with GM-CSF at 1 ng/ml did not change significantly relative to that observed in cells incubated with cytostatic drugs alone. GM-CSF applied at 10 ng/ml in combination with mitoxantrone reduced the cytotoxic effect to 23% colonies as compared with the 20% value recorded for cells incubated with mitoxantrone alone ($P < 0.05$), as is shown in Fig. 2 and

Table 2 Toxic effect of cytostatic drugs on normal bone marrow progenitor cells after incubation with growth factors, expressed as percentages of colonies relative to a control incubated with the corresponding growth factor

			DNR		MIT		Ara-C	
		n	Mean	Range	Mean	Range	Mean	Range
CM		15	38	0–103	20	0–80	18	0–87
G-CSF	5.0 ng/ml	7	17*	5–54	12	1–29	26	4–82
G-CSF	50 ng/ml	9	38	0–125	22	0–106	34	0–149
GM-CSF	1.0 ng/ml	7	32	4–102	12	0–44	25	6–80
GM-CSF	10 ng/ml	8	24	2–66	23*	0–66	29	0–100
IL-3	0.5 ng/ml	7	30*	7–82	13	1–43	21	1–82
IL-3	5.0 ng/ml	8	39	1–106	25	0–95	24	0–77

*$P < 0.05$ (Wilcoxon's signed-rank test)

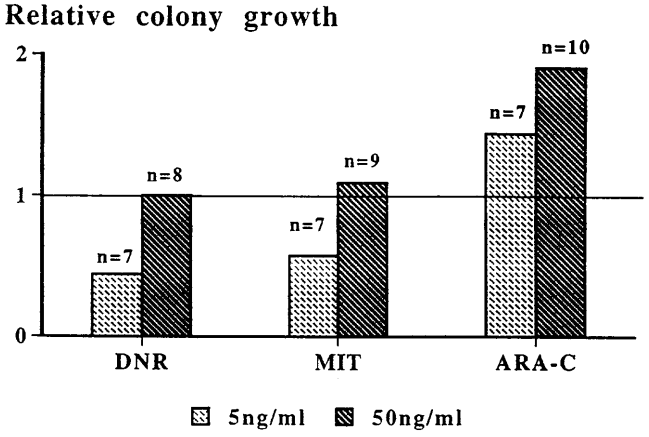

Fig. 1 Effect of G-CSF at 5 and 50 ng/ml on the toxicity of daunorubicin (*DNR*), mitoxantrone (*MIT*), and ara-C to normal hematopoietic progenitor cells. The cytotoxic effect is compared with that observed in controls stimulated with G-CSF. The *horizontal line* indicates the amount of colonies grown after incubation with only cytostatic drugs. *Higher bars* show a decreased effect and *lower bars*, an increased effect of cytostatic drugs applied in combination with G-CSF

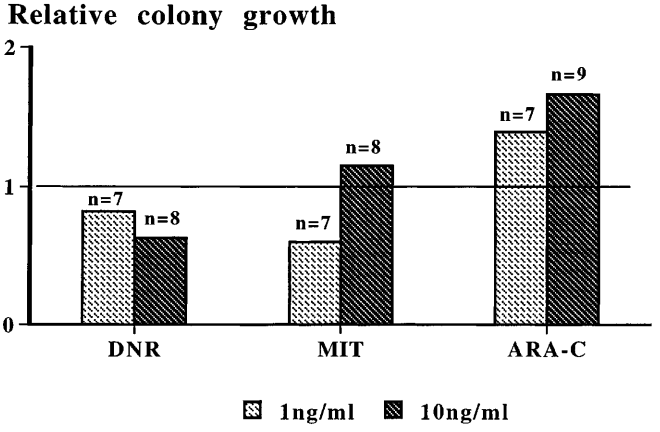

Fig. 2 Effect of GM-CSF at 1 and 10 ng/ml on the toxicity of DNR, MIT, and ara-C to normal hematopoietic progenitor cells. The cytotoxic effect is compared with that observed in controls stimulated with GM-CSF. The *horizontal line* indicates the amount of colonies grown after incubation with only cytostatic drugs. *Higher bars* show a decreased effect and *lower bars* an increased effect of cytostatic drugs applied in combination with GM-CSF

Table 2. Normal hematopoietic progenitor cells incubated with ara-C in combination with GM-CSF at both 1 and 10 ng/ml showed a decrease in toxicity from 18% to 25% and 29%, respectively (nonsignificant).

IL-3 and cytostatic drugs

IL-3 at 0.5 ng/ml induced a significant increase in the toxicity of daunorubicin from 38% to 30% colonies ($P < 0.05$) as compared with an IL-3-stimulated control (Table 2, Fig. 3). An increase in the toxicity of mitoxantrone was also observed after incubation with IL-3 at 0.5 ng/ml (from 20% to 13%; nonsignificant). IL-3 applied at 5 ng/ml in combination with daunorubicin or mitoxantrone did not alter the toxicity of these drugs. The toxicity of ara-C was not altered by IL-3 at either 0.5 or 5.0 ng/ml.

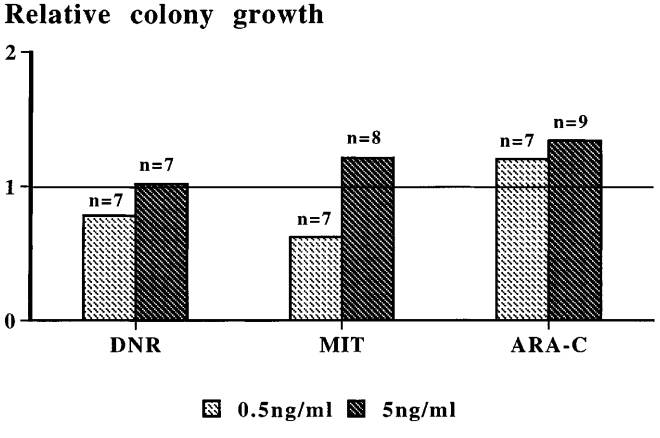

Fig. 3 Effect of IL-3 at 0.5 and 5 ng/ml on the toxicity of DNR, MIT, and ara-C to normal hematopoietic progenitor cells. The cytotoxic effect is compared with that observed in controls stimulated with IL-3. The *horizontal line* indicates the amount of colonies grown after incubation with only cytostatic drugs. *Higher bars* show a decreased effect and *lower bars*, an increased effect of cytostatic drugs applied in combination with IL-3

Discussion

The aim of the present investigation was to compare in vitro how growth factors would affect the toxicity of cytostatic drugs to normal hematopoietic progenitor cells. Due to limitations of material we had to restrict our experimentation to one concentration of each drug. The incubation concentration and time for each drug were chosen to mimic the intracellular concentrations obtained with pharmacokinetic analyses after in vivo infusion of these drugs. We have previously shown that the intracellular concentration achieved after a rapid infusion of daunorubicin at 60 mg/m^2 corresponds to that reached following incubation with a 0.2-μM concentration for 1 h, and for mitoxantrone the concentration reached after incubation at 0.05 μM for 1 h mimics that resulting from a rapid infusion at 12 mg/m^2 [23, 25]. The reason why we did not wash the cells after incubation was that we had previously found that this was the way to mimic the in vivo exposure to and efflux of the drug. In addition, anthraquinones are often given for 2–3 consecutive days. The continuous incubation of ara-C could be regarded as a long-term incubation and could not be transformed to a continuous incubation in vivo. Ara-C is relatively stable and its toxicity increases with time [20].

Incubations with growth factors were carried out at two concentrations, one concentration normally used for in vitro tests [3, 6, 14, 15] and a lower one that was closer to the serum levels obtained in vivo [11, 18]. For

GM-CSF the peak plasma concentration approaches 3.7 ng/ml after administration of a 3 μg/kg dose, and for G-CSF it reaches 10 ng/ml after a dose of 5 μg/kg. Endogenous serum levels of G-CSF, GM-CSF, and IL-3 are generally lower than 0.05 ng/ml [11, 18].

Conditioned medium or growth factors are prerequisites for the growth of control cells. In the present study, all cell cultures, including the controls, underwent a basic stimulation with conditioned medium (CM) produced by a cell line, 5637, which contains a mixture of growth factors [29]. We used the CM as a natural basic stimulator in all the cultures so as to mimic the physiological conditions in a patient. For practical reasons the colonies were counted on either day 10, day 11, or day 12. The number of colonies counted on day 10 was the same as that found on day 12, although the colonies could vary slightly in size.

GM-CSF and IL-3 stimulated the normal bone marrow cells to a higher proliferation rate than was observed in the corresponding untreated controls, but incubation with G-CSF gave no significant increase in colony growth. Sieff [21] has shown that both GM-CSF and IL-3 promote the growth of early multilineage cells and target several types of cells, such as CFU-GM, CFU-mix, erythroid burst-forming units (BFU-B), CFU-G, CSF-M, and CFU-Meg, whereas G-CSF has a predominant effect on CFU-G alone. This may indicate that to a great extent the CM stimulates the same target cells as does G-CSF.

There were pronounced interindividual variations in the sensitivity of the progenitor cells to cytostatic drugs combined with growth factors. This work showed an increase in the toxicity of daunorubicin and, to a smaller extent, of mitoxantrone when incubated with growth factors, whereas the growth factors instead reduced the effect of ara-C. In a previous work we studied how growth factors influenced the effect of cytostatic drugs on leukemic cells [24]. Similarly to the present results, G-CSF increased the toxicity of daunorubicin and mitoxantrone, whereas GM-CSF and IL-3 did not significantly alter the effect of these drugs. In contrast to the results obtained with normal progenitor cells, all growth factors increased the toxic effect of ara-C on leukemic cells.

Several studies have shown that tumor cells become more sensitive to ara-C [1, 14] and daunorubicin [19] after treatment with growth factors. Few reports have been published on alterations in the toxicity of cytostatic drugs to normal hematopoietic progenitor cells recruited into the S phase with GM-CSF, G-CSF, and IL-3 [3, 28]. Waga et al. [28] found an increase in the sensitivity of normal CFU-GM to ara-C but not to daunorubicin after stimulation with growth factors. In our study, normal hematopoietic progenitor cells were more sensitive to daunorubicin after stimulation with G-CSF and IL-3. This finding is in accordance with those reported by Towatari et al. [27], who have claimed that G-CSF increases topoisomerase II levels and, thereby, the toxicity of topoisomerase II-dependent drugs such as daunorubicin. In contrast, GM-CSF and IL-3 protected normal stem cells from mitoxantrone and ara-C toxicity. Bhalla et al. [3, 4] have described that due to the stimulation of normal bone marrow cells with GM-CSF and IL-3 the dCTP pool is expanded. This should inhibit the enzymes crucial for ara-C activation in normal cells but not in leukemic cells. This finding is in accordance with our present results as well as those we previously obtained in leukemic cells [24].

Thus far, no conclusive evidence has been presented for a better remission rate or lower degree of toxicity to normal bone marrow in patients treated with cytokines in combination with chemotherapy [2, 8–10, 22]. Estey et al. [9] have shown a lower remission rate in patients with acute leukemia after treatment with GM-CSF added to daunorubicin and ara-C as compared with the latter drugs alone. Heil et al. [10] found no increased protection of normal bone marrow after the addition of GM-CSF to a combination of daunorubicin ara-C, and etoposide and detected no improvement in the treatment outcome relative to the results obtained using cytostatic drugs alone. In a study in which elderly patients were treated with G-CSF after chemotherapy, Dombret et al. [8] found a higher rate of complete remissions, although there was no decrease in the duration of neutropenia. This finding may be in accordance with our data on daunorubicin, indicating that both the normal and the leukemic hematopoietic stem-cell populations were more heavily damaged when this drug was combined with G-CSF [24]. A higher remission rate after the administration of G-CSF in combination with daunorubicin might indicate that the increased effect of G-CSF and daunorubicin on the tumor cells is more important than the increased toxicity of this combination to normal progenitor cells.

We conclude that on the whole, growth factors exert small effects on the cytocidal effects of myelotoxic drugs on normal bone marrow cells. However, our results indicate an increase in the toxicity of anthracyclines, especially daunorubicin, to normal progenitor cells when these drugs are combined with G-CSF or IL-3 at clinical concentrations.

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