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Variable effects of tamoxifen on human hematopoietic progenitor cell growth and sensitivity to doxorubicin

Karen E. Woods[1], Steven Grant[2], Saul Yanovich[2], David A. Gewirtz[1]

[1] Department of Pharmacology/Toxicology, Medical College of Virginia, Richmond, VA 23298, USA
[2] Department of Medicine, Medical College of Virginia, Richmond, VA 23298, USA

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Abstract. To determine the influence of tamoxifen on the drug sensitivity of normal human hematopoietic progenitor cells, T-cell- and adherent-cell depleted human bone marrow mononuclear cells (T^-, Ad^-) were exposed in vitro to 5 μM tamoxifen for 24 h. The effects of tamoxifen were highly variable, as exposure to tamoxifen produced an increase ($97\% \pm 12.3\%$) in the growth of day-12 committed myeloid progenitors (CFU-GM) in only four of ten experiments utilizing bone marrow from different donors. When T^-, Ad^- myeloid progenitor cells treated with tamoxifen were subsequently exposed to doxorubicin, 7 of 14 experimental samples studied demonstrated a net increase in the number of surviving clonogenic cells as compared with cells exposed to doxorubicin alone. Tamoxifen also stimulated the growth of a more purified ($CD34^+$-selected) progenitor cell population in four of four experiments (by $62.5\% \pm 4.9\%$) but did not increase the survival of these cells upon exposure to doxorubicin; in fact, in five of ten experimental samples, tamoxifen enhanced cell sensitivity to doxorubicin. Taken together, these observations indicate that tamoxifen produces variable stimulation of committed myeloid progenitor cell growth in vitro. Furthermore, while under some circumstances, tamoxifen appears to have the capacity to enhance CFU-GM survival in the presence of doxorubicin, this drug combination may also result in enhanced toxicity to normal bone marrow progenitors.

Introduction

The antiestrogen tamoxifen, a drug used in the treatment of hormone-responsive breast cancer [16], has shown synergistic cytotoxicity with the anthracycline antibiotic doxorubicin against several drug-resistant tumors in vitro, including lymphoblastic and myeloid leukemia cells [2, 25], ovarian tumor cells [5, 10], and breast tumor cells [9]. Recent studies in our laboratories have confirmed these findings in the multidrug-resistant MCF-7 breast-tumor cell line (Woods et al., unpublished data), and Leonessa et al. [18, 19] have reported interaction of tamoxifen with the multidrug-resistance pump. Evidence has also been presented suggesting that the multidrug transporter is present in a variety of human host tissues [30], including primitive hematopoietic cells [4, 20], raising the possibility that such a mechanism might serve to protect these cells from anthracycline-mediated toxicity. If this is the case, tamoxifen might increase the sensitivity of normal human hematopoietic progenitors to doxorubicin and to other drugs that are substrates for the multidrug-resistance pump. However, there is currently no information available concerning the effect of exposure to pharmacologically relevant concentrations of tamoxifen on the in vitro sensitivity of normal committed myeloid progenitors (CFU-GM) to antitumor drugs. To address this issue, the effects of tamoxifen on sensitivity to doxorubicin were compared in both T-cell- and adherent-cell-depleted bone marrow mononuclear cells and in $CD34^+$ selected progenitors. The latter population was chosen because previous studies have indicated that $CD34^+$-selected cells may represent a purer population with respect to expression of the multidrug-resistance phenotype [4].

Materials and methods

Chemicals. Doxorubicin (Adriamycin) and tamoxifen citrate were obtained from Sigma Chemical Co. (St. Louis, Mo.). Doxorubicin was dissolved in deionized water and tamoxifen in ethanol. Drug solutions were filtered through an Acrodisc 0.45-μm syringe filter (Gelman Sciences, Ann Arbor, Mich.) to achieve sterility and were stored as frozen stock solutions.

Bone-marrow cell separation. Bone marrow aspirates were obtained from the posterior iliac crests of normal paid volunteers. Informed

Abbreviations: CFU-GM, granulocyte-macrophage colony-forming units; T^-, Ad^-, T-cell- and adherent-cell-depleted bone marrow mononuclear cells

Correspondence to: Dr. David A. Gewirtz, Department of Medicine, Medical College of Virginia, Box 230 MCV Station, Richmond, VA 23298, USA

consent was obtained and the studies described herein were approved by the Human Investigations Committee of the Medical College of Virginia. Specimens were aspirated into sterile syringes containing 100 IU of preservative-free heparin (Bristol-Myers Squibb Company, New York, N.Y.) diluted 1:5 with 1X McCoy's 5a medium (Gibco, Grand Island, N.Y.), centrifuged over lymphocyte separation medium (Ficoll-Hypaque gradient; Organon Teknika, Durham, N.C.) to isolate the mononuclear cell fraction, and depleted of T lymphocytes and adherent cells (T^-, Ad^-) by sheep red-blood-cell rosetting and plastic adherence as described previously [7, 21].

Isolation of purified progenitor cells, CD34+. T-cell and adherent-cell-depleted (T^-, Ad^-) bone marrow mononuclear cells were obtained as described above and further selected for purified hematopoietic progenitor cells (CD34+-selected) utilizing the previously described modification [12, 13] of the immune panning technique of Engleman et al. [6]. Briefly, nonadherent mononuclear cells were suspended in serum-free 1X McCoy's 5a medium at a density of 10^7 cells/ml and were exposed to 20 μl/10^6 cells of anti-HPCA-1 (anti-human progenitor cell antigen, anti-CD34; Becton-Dickinson, Mountainview, Calif.) at 4° C for 1 h. The cells were gently pelleted, washed once with 1X McCoy's 5a medium containing 0.2% bovine serum albumin, and resuspended at a cell density of 5×10^6 cells/ml. At this time, cells were incubated with magnetic dynabeads (M-450; Dynal Incorporated, Great Neck, N.Y.) covalently bound to goat anti-mouse IgG; a cell:bead ratio of 35:1 was utilized. The cell/bead mixture was vortexed twice for approximately 30 s and centrifuged at 400 *g* for 10 min at 4° C, and the pellet was resuspended in the original volume of medium prior to agitation for 30 min at 4° C. Cells bound to the beads were removed with a magnetic particle concentrator. The yield of $CD34^+$ cells was approximately 1% of the original mononuclear population, with approximately 97% purity being determined by flow cytometric analysis. The $CD34^+$-bearing cells were suspended in medium containing 10% fetal bovine serum (Hyclone, Logan, Utah) prior to cloning in soft agar as described below.

CFU-GM assay. T-cell- and adherent-cell-depleted bone marrow mononuclear cells (T^-, Ad^-) were suspended in 1X McCoy's 5a medium containing 10% fetal bovine serum at a cell density of 1×10^6 cells/ml and were exposed to 5 μ*M* tamoxifen for 24 h at 37° C in a fully humidified incubator containing 5% CO_2. This period as well as the 2-hour exposure to doxorubicin (see below) were chosen for consistency with other studies in progress designed to utilize tamoxifen in combination with doxorubicin to eliminate multidrug-resistant MCF-7 breast tumor cells (Woods et al., unpublished data). Exposure to tamoxifen was not found to influence the number of mononuclear cells in suspension in relation to untreated controls (data not provided). In studies utilizing doxorubicin, cells were incubated with doxorubicin for an additional 2 h in the continued presence of tamoxifen, after which they were washed, the cell density was normalized, and cells were plated in soft agar in tissue-culture plates (Corning, Corning, N.Y.) as previously described [16]. T^-, Ad^- mononuclear cells were plated at a density of 2×10^4 cells/plate. Studies utilizing $CD34^+$-isolated progenitors were performed in a similar fashion utilizing a treatment density of $1.5-3\times10^4$ cells/plate and a plating density of 4×10^3 cells/plate. In all cases, 10% 5637 conditioned medium (filtered and stored frozen at –20° C) derived from confluent cultures of the bladder-tumor cell line 5637 (ATCC, Rockville, MD) was utilized as a source of colony-stimulating factors [3].

At the end of a 12-day incubation period, colonies consisting of groups of 40 or more cells of granulocyte- or macrophage-like appearance were scored with an inverted microscope (Olympus CK2, Olympus, Japan). The studies described in this report were performed utilizing a CFU-GM assay because it has been reported that the survival of these progenitors is predictive of hematologic reconstitution following bone marrow purging [27].

Statistical analyses. Data were analyzed using Student's *t*-test for unpaired variables. *P* values of <0.05 were considered significant.

Table 1. Influence of tamoxifen on growth of human progenitor cells

Experiment	% Control	*P* value	Gender
Donor A	125±14.8	<0.2	M
Donor B	183±4.3	<0.02	M
Donor C	188±10.7	<0.01	F
Donor D	115±9.9	<0.9	M
Donor E	107±11.1	<0.9	M
Donor F	116±6.3	<0.2	M
Donor G	229±5.0	<0.001	M
Donor H	189±6.4	<0.001	M
Donor I	92±3.2	<0.4	M
Donor J	101±4.1	<0.9	M

T-cell- and adherent-cell-depleted bone marrow progenitor cells were obtained as described in Materials and methods and incubated in the presence of 5 μ*M* tamoxifen for 24 h at a density of 1×10^6 cells/ml. Cells were washed and counted and the CFU-GM clonogenic assay was performed as described. Values indicate the mean percentage of control growth ± SE for triplicate determinations in each experiment. The plating efficiency in the absence of tamoxifen was 0.5%; 28 individual control samples produced an average of 93 colonies after plating

Table 2. Influence of tamoxifen on CFU-GM after exposure to doxorubicin (% Control Growth)

Experiment	0.05 μ*M* DOX	0.1 μ*M* DOX	0.5 μ*M* DOX	1 μ*M* DOX	Gender
Donor A	59±1.2 (81±4.8)*	28±2.9 (77±5.2)*	–	13±1.8 (29±1.8)*	M
Donor B	29±6.7 (127±0.0)*	68±1.0 (100±10)	13±5.4 (136±12.5)*	10±5 (198±0.0)*	M
Donor C	78±10 (115±20)	40±5 (33±1.7)	2±1 (25±6.7)*	2±1 (4±3)	F
Donor G	119±13.1 (167±14.7)	90±13.6 (110±6.4)	67±8.7 (74±2.1)	–	M

T-cell- and adherent cell-depleted progenitors were obtained as described in Materials and methods and incubated in the absence or presence of 5 μ*M* tamoxifen for 24 h prior to exposure to varied concentrations of doxorubicin (DOX) for 2 h (in the absence or continued presence of tamoxifen, respectively). Clonogenicity was determined after 12 days. Donor samples correspond to those presented in Table 1. Values indicate the mean percentage of control growth ± SE for triplicate determinations in each experiment. Values in parentheses are for samples preincubated with tamoxifen

* Significance with a *P* value of <0.05

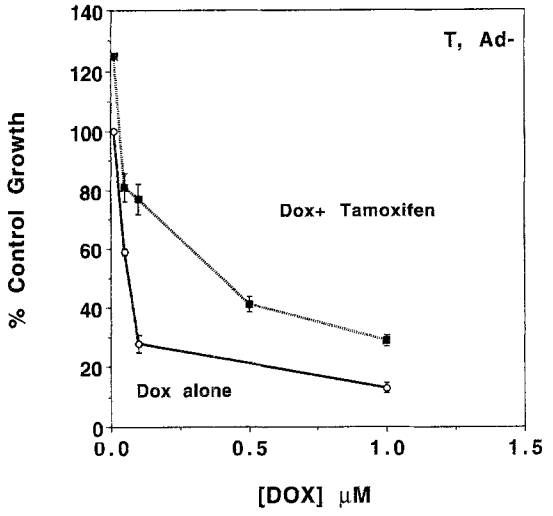

Fig. 1. T⁻, Ad⁻ human bone marrow mononuclear cells were obtained and processed as described in Materials and methods and incubated in the absence or presence of 5 μ*M* tamoxifen for 24 h prior to exposure to doxorubicin (in the absence or the continued presence of tamoxifen, respectively) for 2 h. Clonogenicity was determined after 12 days. Values indicate the mean percentage of CFU-GM control growth ± SE for triplicate determinations in a single experiment. Note that Fig. 1 is also presented as donor A in Table 2

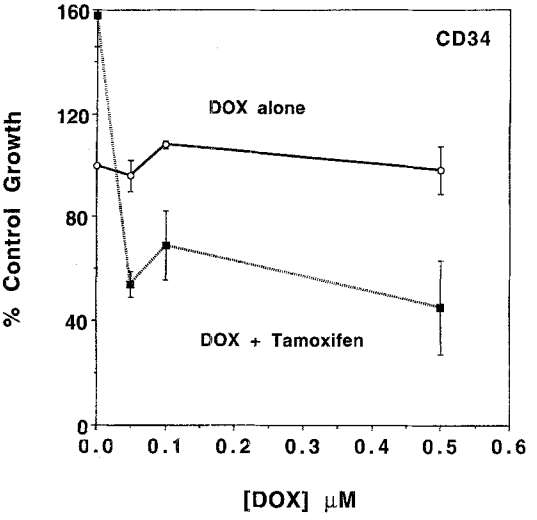

Fig. 2. CD34⁺ isolated human bone marrow progenitor cells were obtained and processed as described in Materials and methods and incubated in the absence or presence of 5 μ*M* tamoxifen prior to exposure to doxorubicin (in the absence or the continued presence of tamoxifen, respectively) for 2 h. Clonogenicity was determined after 12 days. Values represent the mean percentage of CFU-GM control growth ± SE for triplicate determinations within a single experiment. Note that Fig. 2 is also presented as donor K in Table 4

Results

Table 1 presents the result of ten separate experiments assessing the influence of tamoxifen on the growth of T⁻, Ad⁻ human bone marrow progenitor cells obtained from different donors. In four of ten experiments (B, C, G, and H), a statistically significant increase was observed in the number of colonies formed after treatment with tamoxifen, the average increase being 97%±12.3%. In the remaining experiments, no significant change was noted. In two additional studies, treatment of T⁻, Ad⁻ bone marrow progenitor cells with 1 μ*M* tamoxifen for 24 h stimulated the proliferative capacity by 23.0%±14.0% (data not shown).

Table 3. Influence of tamoxifen on growth of CD34⁺ progenitor cells

Experiment	% Control	*P* value	Gender
Donor K	158±12.4	<0.05	F
Donor L	169±7.4	<0.01	F
Donor M	152±5.5	<0.01	F
Donor N	170±27.5	<0.01	M

CD34⁺ isolated progenitors were obtained as described in Materials and methods. Cells were incubated in the presence of 5 μ*M* tamoxifen for 24 h, washed, and counted, and the CFU-GM assay was performed as described. Values indicate the mean percentage of control growth ± SE for triplicate determinations within each experiment. The plating efficiency in the absence of tamoxifen was approximately 2.6%; 12 individual control samples produced an average of 106 colonies after plating

Treatment of T⁻, Ad⁻ bone marrow progenitor cells with doxorubicin alone produced concentration-dependent cytotoxic effects that varied between the different donor samples (Table 2). The 90% growth-inhibitory concentration (IC_{90} value) generally fell between 0.5 and 1 μ*M*, which is somewhat lower than the values of approximately 3 and 1.8 μ*M* reported by Auber et al. [1] and Speth et al. [29], respectively. Figure 1 shows the results of a representative study (also presented as condition A in Table 2) in which T⁻, Ad⁻ human bone marrow progenitor cells exposed to doxorubicin following a 24-h pretreatment with 5 μ*M* tamoxifen demonstrated increased survival as compared with cells exposed to doxorubicin alone. Table 2 indicates that there was considerable variability in the extent of this effect; although an increase in survival was noted in all but one experimental sample (donor C at 0.1 μ*M* doxorubicin), this increase was statistically significant in only 50% of the experimental samples.

In separate studies, it was demonstrated that the vehicle in which the tamoxifen was dissolved (final concentration of 0.05% ethanol) did not significantly alter the growth of CFU-GM derived from T⁻, Ad⁻ hematopoietic progenitor cells (103.7%±5.7% of control growth in three experiments), nor did it alter doxorubicin toxicity (data not shown).

These observations indicating increased survival of bone marrow progenitor cells exposed to doxorubicin following pretreatment with tamoxifen are contrary to what might be expected in view of the theoretical capacity of tamoxifen to sensitize these cells to doxorubicin through inhibition of the multidrug-resistance pump [4]. In addition, stimulation of growth might also be expected to enhance sensitivity to doxorubicin. Consequently, studies were undertaken to assess the influence of tamoxifen on the sensitivity to doxorubicin of a more purified (CD34⁺-isolated) progenitor cell population in which the potential influence of an accessory cell population might be minimized. Table 3 presents the results of four separate studies indicating that 5 μ*M* ta-

Table 4. Influence of tamoxifen on CFU-GM after exposure of doxorubicin

Experiment	0.1 μ*M* DOX	0.5 μ*M* DOX	1 μ*M* DOX	Gender
Donor K	96±6.0 (54±5.0)*	108±1.3 (69±13.5)*	98±9.2 (45±18.0)*	F
Donor L	62±8.0 (49±7.2)	31±3.0 (38±2.5)	–	F
Donor M	123±11.3 (114±6.6)	86±9.8 (29±3.6)*	–	F
Donor N	105±7.3 (89±6.5)	71±8.6 (67±4.8)	9±3.1 (1±0.95)*	M

$CD34^+$-isolated progenitors were obtained as described in Materials and methods and incubated in the presence or absence of 5 μ*M* tamoxifen for 24 h prior to exposure to varied concentrations of doxorubicin for 2 h (in the absence or continued presence of tamoxifen, respectively). Clonogenicity was determined after 12 days. Donor samples correspond to those presented in Table 3. Values indicate the mean percentage of control growth ± SE for triplicate determinations in each experiment. Values in parentheses are for samples preincubated with tamoxifen

* Significance with a P value of <0.05

moxifen stimulated the growth of CFU-GM derived from $CD34^+$ bone marrow mononuclear cells by 62.3%±5.0% in experiments employing cells from different donors. The experiment shown in Fig. 2 (also presented as condition K in Table 4) demonstrates that in contrast to the results obtained using the T-cell- and adherent-cell-depleted mononuclear cells, tamoxifen pretreatment sensitized $CD34^+$-isolated progenitor cells to doxorubicin. (Interestingly, the cells from the donor in Fig. 2 were relatively insensitive to doxorubicin, attesting to the variability in responsiveness to doxorubicin.) Table 4 summarizes the results of four studies employing $CD34^+$-isolated cells and indicates that the effects of tamoxifen on progenitor cell sensitivity to doxorubicin were variable; that is, whereas there was a net decrease in the survival of CFU-GM (after exposure to tamoxifen + doxorubicin as compared with exposure to doxorubicin alone) in all but one experimental sample (donor L, 0.5 μ*M* doxorubicin), this decrease was statistically significant in only 50% of the experimental samples.

Discussion

T^-, Ad^- cells

The present studies demonstrate a high degree of variability in the response of bone marrow progenitor cells to tamoxifen in vitro. In experiments using T^-, Ad^- bone marrow progenitor cells, CFU-GM from some donors showed an unequivocal increase in proliferative capacity, whereas there was no significant effect on CFU-GM from other individuals. Variable responses to tamoxifen were also observed in assessments of its effects on progenitor sensitivity of T^-, Ad^- bone marrow mononuclear cells to doxorubicin; increased survival was quite pronounced in isolated hematopoietic progenitor cells from some donors but was barely detectable in progenitor cells obtained from others. The variable capacity of tamoxifen to stimulate progenitor cell growth and to alter sensitivity to doxorubicin raises the possibility that the level of a putative estrogen receptor on hematopoietic cells capable of recognizing tamoxifen [11, 15] may differ markedly within the general population. In this regard, hematopoietic cells of the monocyte-macrophage linkage have been found to express the estrogen (E_2) receptor [14].

Additionally, differences in the response of CFU-GM derived from T^-, Ad^- bone marrow progenitors to tamoxifen may be due to differences in the influence of populations of residual accessory cells. Since tamoxifen should theoretically sensitize progenitor cells at the level of the multidrug-resistance pump [2, 4], it is possible that residual accessory cells may antagonize the interaction of tamoxifen with the multidrug-resistance pump in T-cell- and adherent-cell-depleted progenitors. This hypothesis is consistent with the results obtained in earlier studies by other investigators [8, 28, 33], which indicated that the calcium channel antagonist verapamil, an agent that interferes with drug efflux via the multidrug-resistance pump [24, 26], failed to enhance the toxicity of doxorubicin toward a heterogeneous population of bone marrow progenitor cells.

The mechanism by which tamoxifen stimulates the in vitro growth of normal human progenitors from a subset of donors awaits definition. It has recently been reported that 17β-estradiol inhibits the production of interleukin-6 [11], a cytokine involved in hematopoiesis [22]. Consequently, tamoxifen may play a permissive role in the elaboration of specific growth-stimulatory factors by bone marrow progenitor cells. Alternatively, tamoxifen may act as an inhibitor of protein kinase C [17, 23] or may antagonize certain differentiation-related events and promote proliferative responses, as has been suggested by earlier studies [21].

$CD34^+$ cells

The studies assessing the effects of 5 μ*M* tamoxifen on the growth of CFU-GM derived from $CD34^+$ progenitors indicate a more homogeneous response in the more highly enriched myeloid progenitor cell population, although the sample size utilized in these studies was significantly smaller (it is possible that a more heterogeneous response would be observed with a larger sample size). Tamoxifen produced a statistically significant increase in cell number in four of four individual samples of CFU-GM utilizing $CD34^+$-isolated progenitors from different donors. However, when sensitivity to doxorubicin was evaluated in tamoxifen-pretreated $CD34^+$-isolated progenitors, a heterogeneous response was again noted; enhanced sensitivity to doxorubicin was observed in only 50% of the experimental samples (in contrast to the enhanced *survival* promoted by tamoxifen in CFU-GM derived from T^-, Ad^- bone marrow mononuclear cells exposed to doxorubicin).

Since CFU-GM are presumably derived from identical progenitor cells within both T-cell- and adherent-cell-depleted and $CD34^+$-isolated cell populations, the differing effects of tamoxifen on doxorubicin sensitivity in these

progenitors suggest that accessory cell populations may play a role in modifying at least some of the effects of tamoxifen on doxorubicin responsiveness. However, because of the possibility of multiple complex interactions, the precise nature of these modifications is difficult to determine without extensive dissection of the contributions of different cell populations.

The observation that tamoxifen appears to sensitize CFU-GM derived from CD34+ bone marrow progenitor cells to doxorubicin is consistent with a role for this agent in inhibiting the multidrug-resistance pump in both normal and neoplastic cells [2, 4, 5, 8–10, 18, 19, 25]. It also suggests that the multidrug-resistance pump may play an important role in regulation of drug efflux in the marrow of at least some individuals within the general population. It is possible that the variability of the response to tamoxifen in combination with doxorubicin indicates heterogeneous expression of the multidrug-resistance phenotype. Sensitization to doxorubicin by tamoxifen does not appear to be related to its growth-stimulatory actions since a similar effect was not observed in CFU-GM derived from the less purified (T^-, Ad^-) progenitor cell population in which growth stimulation was also observed.

Clinical ramifications

It is not immediately apparent whether the studies utilizing CFU-GM derived from T-cell- and adherent-cell-depleted progenitors or those utilizing CFU-GM derived from the CD34+-isolated population will be more predictive of the nature of drug interaction in vivo. Consequently, although the in vivo significance of these in vitro findings is unknown, it is noteworthy that clinical trials employing tamoxifen in conjunction with agents susceptible to the multidrug-resistance pump have recently been initiated [32]. In these studies, dose-limiting neurotoxicity was noted in patients treated with tamoxifen; however, there was no evidence of enhanced vinblastine-induced myelotoxicity as mediated by tamoxifen. The studies described in this communication suggest that such trials should be approached with caution, since there is a theoretical risk that such combinations might be associated with enhanced toxicity to normal bone marrow progenitors. In this context, Tormey et al. [31] suggested slightly enhanced myelotoxicity arising from the use of these drugs in combination. It is conceivable that a better understanding of the complex factors responsible for tamoxifen-mediated modulation of myelosuppression produced by different antineoplastic drugs might lead to safer and more effective combination regimens in the treatment of human malignancies.

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