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Protracted Low-Dose Effects on Human Endothelial Cell Proliferation and Survival *in Vitro* Reveal a Selective Antiangiogenic Window for Various Chemotherapeutic Drugs[1]

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ABSTRACT

Recent preclinical studies have shown that frequent administration *in vivo* of low doses of chemotherapeutic drugs ("metronomic" dosing) can affect tumor endothelium and inhibit tumor angiogenesis, reducing significant side effects (*e.g.*, myelosuppression) involving other tissues, even after chronic treatment. This suggests that activated endothelial cells may be more sensitive, or even selectively sensitive, to protracted ("high-time") low-dose chemotherapy compared with other types of normal cells, thus creating a potential therapeutic window. To examine this hypothesis, we assessed the effects of several different chemotherapeutic drugs—namely paclitaxel, 4-hydroperoxycyclophosphamide, BMS-275183 (an oral taxane), doxorubicin, epothilone B (EpoB) and its analogue 5-methylpyridine EpoB—on human microvascular or macrovascular endothelial cells, fibroblasts, and drug-sensitive or multidrug-resistant breast cancer cell lines in cell culture, using both short-term (24 h) *versus* long-term (144 h), continuous exposures, where drug-containing medium was replaced every 24 h. Whereas little differential and only weak effects were observed using the short-term exposure, a striking trend of comparative vascular endothelial cell hypersensitivity was induced using the continuous long-term exposure protocol. Potent differential growth inhibition effects as well as induction of apoptosis were observed with IC_{50} values in the range of 25–143 pM for paclitaxel, BMS-275183, EpoB, and 5-methylpyridine-EpoB. In contrast, the IC_{50} values for tumor cells and fibroblasts tested were in the range of 500 pM to >1 nM for these drugs. Similar differential IC_{50} values were noted using 4-hydroperoxycyclophosphamide. The results are consistent with the possibility that continuous low-dose therapy with various chemotherapeutic drugs may have a highly selective effect against cycling vascular endothelial cells, and may be relevant to the use of continuous or frequent administration of low doses of certain types of drugs as an optimal way of delivering antiangiogenic therapy.

INTRODUCTION

Among the many drugs capable of inhibiting angiogenesis in various preclinical models are conventional cytotoxic chemotherapeutic drugs (1). The first report of the antiangiogenic properties of such drugs appeared more than 15 years ago (2), and since then most drugs belonging to virtually every class of anticancer chemotherapeutic agents have been shown to have antiangiogenic effects (1). The basis for these effects, at least in part, is presumably related to the presence of cycling endothelial cells in newly forming blood vessel capillaries (3). Interest in exploiting chemotherapeutic drugs for their antiangiogenic properties in the context of cancer therapy was stimulated recently by two reports (4, 5) using frequent administration of drugs such as cyclophosphamide or vinblastine *in vivo* at doses much lower than the MTD.[3] The rationale for this approach—called antiangiogenic or metronomic "chemotherapy" (4, 6)—is that damage to the tumor's vasculature using conventional regimens can be largely repaired during the long breaks (*e.g.*, 3 weeks) between successive cycles of MTD chemotherapy. Such breaks are normally required to allow for recovery from some of the harmful side effects of such therapies, such as myelosuppression (4). Shortening the time between cycles minimizes the efficacy of the repair process, which requires the use of lower doses of drug. Whereas this strategy seems to enhance the efficacy of the antiangiogenic effects of chemotherapy, in theory it should enhance the undesirable side effects of chemotherapy as well, including myelosuppression. Interestingly, this does not seem to be the case (4, 5, 7, 8) and implies that activated vascular endothelial cells may be more sensitive to certain low, or lower doses, of various chemotherapeutic drugs compared with other types of normal cells, or cancer cells, when exposed in a frequent or continuous manner.

Some preliminary evidence for such a preferential sensitivity of endothelial cells has been suggested on the basis of certain *in vitro* experiments. Thus, several groups have reported that very low (*e.g.*, nanomolar) concentrations of certain drugs such as PTX (7, 9), topotecan, campthothecin (10), or vinblastine (5, 7, 11) can significantly block endothelial cell growth, but not necessarily tumor cell growth *in vitro*. In addition, Vacca *et al.* (11) found that nanomolar or even picomolar concentrations of vinblastine, which did not cause significant antiproliferative effects against endothelial cells, could nevertheless suppress angiogenesis and certain endothelial cell functions considered relevant to angiogenesis, such as migration and protease production. Differential antiproliferative effects of chemotherapeutic drugs on vascular endothelial cells *versus* tumor cells have been noted with vinblastine, PTX, cisplatinum, and adriamycin (7).

All of the aforementioned *in vitro* experiments usually involved a single drug exposure of between 24 and 72 h. However, this does not mirror the *in vivo* situation when protracted low-dose metronomic chemotherapy protocols are administered frequently (*e.g.*, weekly; Ref. 4) every 3 days (5), or even daily (8, 12). We, therefore, asked whether protracted exposures *in vitro* to certain chemotherapeutic drugs might reveal new and important aspects of the antiendothelial cell effects of low-dose metronomic chemotherapy regimens. The purpose of this study was to examine this question and, more specifically, to determine whether there is an "antiangiogenic window" for antineoplastic chemotherapeutic drugs in which drug activity, at comparatively low concentrations, is largely or specifically restricted to endothelial cells. To do so, we designed long-term *in vitro* assays in which human tumor cells, dermal fibroblasts, and macrovascular or microvascular endothelial cells were exposed daily, for up to 6 days,

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[3] The abbreviations used are: MTD, maximum tolerated dose; EpoB, epothilone B; 5-MP-EpoB, 5-methylpyridine EpoB; 4-HC, 4-hydroperoxycyclophosphamide; DXR, doxorubin; PTX, paclitaxel; HUVEC, human umbilical vein endothelial cell(s); HMVEC-d, human dermal microvascular endothelial cell(s); NHDF, normal human dermal fibroblast; VEGF, vascular endothelial growth factor; FBS, fetal bovine serum.

to various low concentrations of different, well known and novel chemotherapeutic drugs (Fig. 1), including 4-HC, PTX, an oral active taxane called BMS-275183 (13), DXR, EpoB (14), and the analogue 5-MP-EpoB (15). Our results show that vascular endothelial cells are, indeed, preferentially affected for properties such as proliferation and induction of apoptosis, by low concentrations of most of these drugs when exposed continuously for protracted periods of time (*e.g.*, 6 days), but not for shorter periods of time (*e.g.*, 24 h).

MATERIALS AND METHODS

Drugs. BMS-275183 (Bristol Myers-Squibb Co.) was provided by Drs. R. Malik and W. Rose. EpoB and 5-MP-EpoB were synthesized by the Nicolaou group (14, 16). 4-HC was a gift from Dr. M. Colvin (Duke University, Durham, NC). DXR and PTX were purchased from Sigma Chemical Co. To perform *in vitro* studies the compounds were stocked in 10 mM solutions: BMS-275183, EpoB, 5-MP-EpoB, and PTX were dissolved in 100% DMSO; and DXR was dissolved in distilled water. 4-HC was stocked as a powder at −20°C and dissolved for every treatment at 4°C in distilled water. All of the drugs were diluted in culture medium immediately before their use.

Cells and Culture Conditions. HUVEC, HMVEC-d, and NHDFs were purchased from Clonetics. The human breast cancer cell line MDA-MB-435 was obtained from Dr. J. Lemontt (Genzyme Corp., Cambridge, MA); and its P-glycoprotein-positive multidrug-resistant variant of MDA-MB-435, called T0.1, was obtained from Dr. D. Cohen (Novartis) and was derived by *in vitro* exposure to increasing concentrations of PTX.

The HUVEC and HMVEC-d cells were maintained in MCDB131 culture medium (JRH Biosciences) supplemented with 10% heat-inactivated FBS (Life Technologies, Inc.), L-glutamine (2 mM; Life Technologies, Inc.), heparin (10 units/ml; Wyeth-Ayerst), epidermal growth factor (10 ng/ml; Upstate Biotechnology Inc.), and basic fibroblast growth factor (5 ng/ml; R&D Systems, Inc.). Human endothelial cells were routinely grown in 1% gelatin-coated tissue culture flasks (Nunc A/S). NHDFs were cultured in 5% FBS RPMI medium (Life Technologies, Inc.) supplemented with L-glutamine; the breast cancer cell lines were maintained and expanded as monolayer culture in 10% FBS DMEM (Life Technologies, Inc.) supplemented with L-glutamine; the T0.1-resistant variant was grown with the addition of 0.1 μM PTX. Cells were kept in a humidified atmosphere of 5% CO_2 at 37°C and harvested with a solution of 0.25% trypsin-0.03% EDTA (Life Technologies, Inc.) when they were in log phase of growth, and maintained at the above-described culture conditions.

Cell Proliferation Assay. *In vitro* chemosensitivity testing was performed on single-cell suspensions of HUVEC, HMVEC-d, NHDF, MDA-MB-435, and T0.1 cells plated in 96-well plastic plates (1% gelatin coated for the endothelial cells) and allowed to attach overnight. Each drug concentration was represented by at least 10 wells and replicated three times. Cells were treated for 24 h (3×10^3 cells/well in 200 μl of medium) or 144 h (1×10^3 and 0.5×10^3 cells/well of normal and cancer cells, respectively, in 200 μl of medium) with PTX (1–1,000 pM), BMS-275183 (1–1,000 pM), EpoB (1–1,000 pM), 5-MP-EpoB (1–1,000 pM), 4-HC (0.1–10,000 nM), and DXR (1–1,000 nM) (Fig. 1); to maintain a constant concentration of the drugs during the protracted 144-h period of the experiments, every 24 h the medium was removed and fresh solutions were added with new medium (Fig. 1). The 4-HC treatments were performed as recommended by Flowers *et al.* (17) to avoid the toxic effects of volatile metabolites. At the end of the experiment, cells were pulsed for 6 h with 2 μCi/well of methyl-[^{3}H]-thymidine (Amersham Life Science), as described previously by Klement *et al.* (5). The concentration of drugs that reduced cell proliferation by 50% (IC_{50}) as compared with controls was calculated by nonlinear regression fit of the mean values of the data obtained in triplicate experiments.

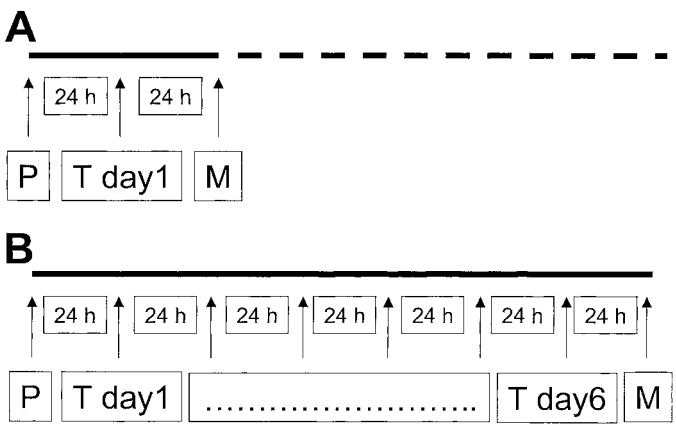

Fig. 1. Experimental design of cell proliferation and apoptosis assays. *A*, single low-dose exposure to chemotherapeutic drugs (24 h). *B*, protracted continuous low-dose exposure to chemotherapeutic drugs (144 h). *P*, plate; *T*, treat; *M*, measure.

Apoptosis Measurements. HUVEC, HMVEC-d, MDA-MB-435, T0.1, and NHDF cells were plated in 100-mm sterile dishes and continuously treated for 144 h, as described above, with 100 pM PTX, 100 pM BMS-275183, 100 pM EpoB, 100 pM 5-MP-EpoB, 100 nM 4-HC, and vehicle alone. At the end of the sixth day of treatment, cells were collected and the Cell Death Detection ELISA Plus kit (Roche) was used to quantify apoptosis as described previously (18) All of the absorbance values (measurement wavelength, 405 nm; reference wavelength, 490 nm) were plotted as a percentage of apoptosis relative to control cells (vehicle only), which were labeled as 100%. All experiments were repeated two times with at least two replicates/sample.

Statistical Analysis. The results (mean values ± SE) of cell proliferation, adhesion, and migration assays were subjected to statistical analysis by ANOVA, followed by the Student-Newman-Keuls test, using the GraphPad Prism© software package (version 3.0; GraphPad Software Inc.). The level of significance was set at $P < 0.05$.

RESULTS

Protracted Low-Dose Treatment with Taxanes, EpoB, and 4-HC Specifically or Preferentially Inhibits Endothelial Cell Proliferation. When endothelial cells (both macrovascular umbilical vein and dermal microvascular) were exposed to the low-dose schedule for only 24 h, significant inhibition was only observed at the highest concentrations of drug tested (*e.g.*, BMS-275183 46.9 ± 4.5%, and EpoB 33.4 ± 2.2% *versus* HUVEC control proliferation, $P < 0.05$; Fig. 2, *left*). In striking contrast, a strong and dose-dependent antiproliferative effect (including at the lower concentrations) was observed when endothelial cells were continuously exposed over 144 h (Fig. 2, *right*). PTX affected only endothelial cells from concentrations of 10 pM (77.3 ± 12% *versus* HMVEC-d control proliferation, $P < 0.05$; Fig. 2*A*, *right*) to 1 nM, which showed maximum inhibition (HUVEC 14.7 ± 2.1%, HMVEC-d 9.6 ± 3.5% *versus* control proliferation, $P < 0.05$; Fig. 2*A*, *right*), with a calculated IC_{50} of 51 and 96 pM for HMVEC-d and HUVEC, respectively. At the same low concentration, both breast cancer cell lines (parental and drug-resistant variant) showed no significant response, whereas the normal dermal fibroblasts were affected at 1 nM. This same trend was observed with the oral taxane BMS-275183 (Fig. 2*B*, *right*) with a maximum inhibition of endothelial cell growth at 1 nM (HUVEC 6.3 ± 1.3%, HMVEC-d 4.5 ± 2.3% *versus* control proliferation, $P < 0.05$; Fig. 2*B*, *right*) with an IC_{50} of 143 and 102 pM for HUVEC and HMVEC-d, respectively. In this case, there was the exception of a significant effect at the highest dose tested on the parental cancer cell line MDA-MB-435 (7.2 ± 1.5% *versus* control proliferation, $P < 0.05$, $IC_{50} = 498$ pM; Fig. 2*B*, *right*) but not the multidrug-resistant T0.1 variant. Thus, a relative therapeutic antiangiogenic window appeared well defined in this case, particularly in comparison with the PTX-resistant breast cancer cell line variant. EpoB, on the other hand, showed a potent antiproliferative activity at 1 nM after 144 h (Fig. 2*C*, *right*) on all cell lines. In this case, the range of endothelial specificity appears narrow compared with MDA-MB-435 ($IC_{50} = 44$ and 25 pM for HUVEC and HMVEC-d, respectively;

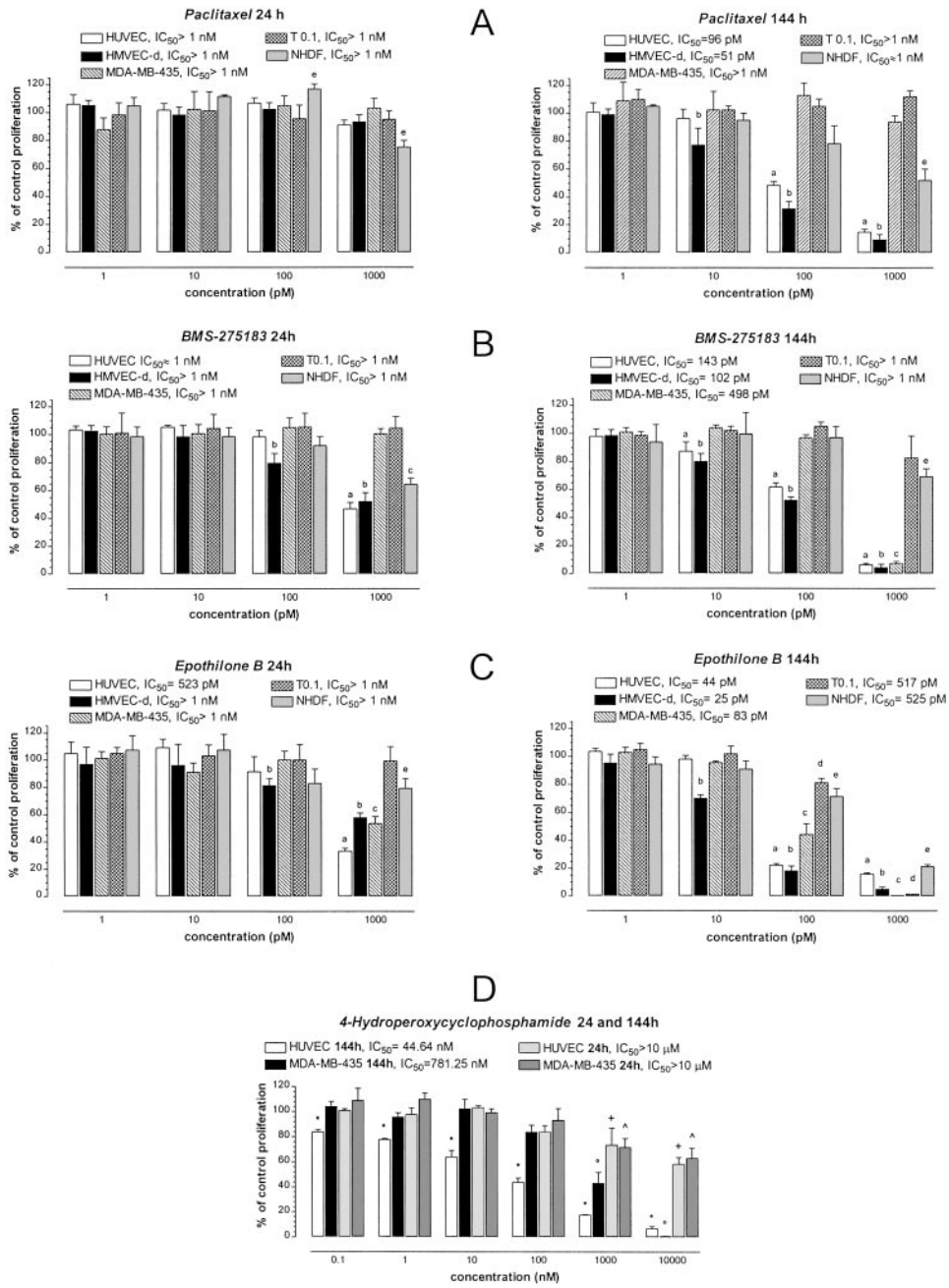

Fig. 2. Effect of low-dose PTX (*A*), BMS-275183 (*B*), EpoB (*C*), and 4-HC (*D*) on *in vitro* cell proliferation. The antiproliferative effects of the drugs were studied using both short-term (24 h, *left*) and prolonged continuous exposures (144 h, *right*) on the HUVEC, HMVEC-d, MDA-MB-435, T0.1, and NHDF cell lines. *Columns* and *bars*, mean values ± SE, respectively. $^{a}P < 0.05$ *versus* HUVEC controls; $^{b}P < 0.05$ *versus* HMVEC-d controls; $^{c}P < 0.05$ *versus* MDA-MB-435 controls; $^{d}P < 0.05$ *versus* T0.1 controls; $^{e}P < 0.05$ *versus* NHDF controls; $*P < 0.05$ *versus* 144 h HUVEC controls; $^{\circ}P < 0.05$ *versus* 144 h MDA-MB-435 controls; $^{+}P < 0.05$ *versus* 24 h HUVEC controls; $\hat{}P < 0.05$ *versus* 24 h MDA-MB-435 controls.

IC_{50} = 83 for MDA-MB-435), but not if compared with T0.1 (IC_{50} = 517 pM); in this case, the difference remained quite significant. In contrast, for all of the compounds tested, the normal human dermal fibroblasts were affected in their proliferation only at the highest concentrations used but never at the levels observed to be effective against endothelial cells (Fig. 2, *right*).

The toxic metabolite/alkylating agent 4-HC, a compound that in solution spontaneously reduces to 4-hydroxycyclophosphamide (the first active metabolite formed in the metabolism of cyclophosphamide), showed a significant dose-dependent inhibition of HUVEC proliferation (Fig. 2*D*, $P < 0.05$). This effect was observed at concentrations that did not affect MDA-MB-435 cancer cell growth. The IC_{50} in the 144-h treatments were 44.64 and 781.25 nM for HUVEC and the MDA-MB-435 cell line, respectively. As shown in Fig. 2*D*, the 4-HC concentrations targeting only endothelial cells were between 1 and 100 nM (*e.g.*, at 1 nM 77.7 ± 1% *versus* control proliferation, at 100 nM 43.7 ± 3.5% *versus* control proliferation; $P < 0.05$), which were in sharp contrast to the MDA-MB-435 breast cancer cell line (at 1 nM 95.6 ± 3.4% *versus* control proliferation, at 100 nM 83.7 ± 5.5% *versus* control proliferation).

Not All Antineoplastic Drugs Have a Specific Antiproliferative Activity Against Endothelial Cells. In contrast to the above mentioned compounds, DXR and 5-MP-EpoB did not show a significant difference in their relative effects on the endothelial and the cancer cell

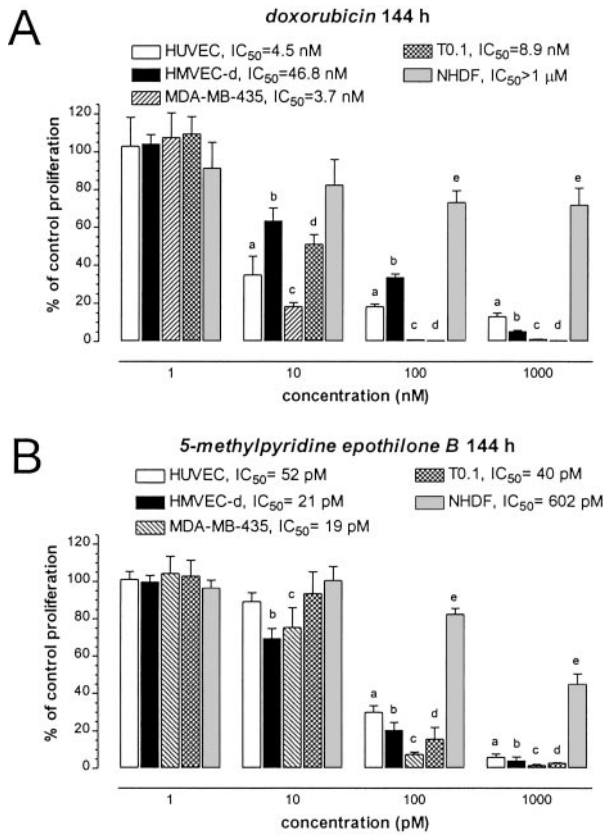

Fig. 3. Effect of low-dose DXR (*A*) and 5-MP-EpoB (*B*) on *in vitro* cell proliferation. The antiproliferative effects of the drugs were studied in the prolonged continuous exposure of 144 h on the HUVEC, HMVEC-d, MDA-MB-435, T0.1 and NHDF cell lines. *Columns* and *bars*, mean values ± SE, respectively. $^{a}P < 0.05$ *versus* HUVEC controls; $^{b}P < 0.05$ *versus* HMVEC-d controls; $^{c}P < 0.05$ *versus* MDA-MB-435 controls; $^{d}P < 0.05$ *versus* T0.1 controls; $^{e}P < 0.05$ *versus* NHDF controls.

lines (Fig. 3, *A* and *B*, respectively) using the low-dose protracted time course regimen. The degree of the antiproliferative activity was the same against endothelial cells and cancer cells (*e.g.*, DXR IC_{50} = 4.5 nM and 3.7 nM for HUVEC and MDA-MB-435, respectively; or 5-MP-EpoB IC_{50} = 21 pM and 19 pM for HMVEC-d and MDA-MB-435, respectively), suggesting that these drugs might act as antitumor compounds by affecting both cancer and endothelial cells at the same drug concentration. These results also show that the selectivity of endothelial cells to the other drugs we tested is not simply a function of decreased "hardiness" of such cells, compared with fibroblasts or tumor cells.

Protracted Low-Dose Chemotherapy *in Vitro* Induces Endothelial Cell Apoptosis. To further investigate the specific effect of treatments on endothelial cells, an ELISA assay was performed to quantify the cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death in treated samples. As shown in Fig. 4, the assay revealed a markedly higher increase of apoptosis in the treated endothelial cells when compared with the cancer cell lines or fibroblasts, after the 6 days of treatment, especially with 100 pM PTX (+210% and +230% for HUVEC and HMVEC-d *versus* controls, respectively) and 100 nM 4-HC (+223% and +211% for HUVEC and HMVEC-d *versus* controls, respectively). In these experiments, 5-MP-EpoB did not show any selective effect on endothelial cells.

DISCUSSION

Our results highlight the importance of the concept of "high-time" chemotherapy (19, 20), *i.e.*, that prolonged exposure times, once an effective dose of drug is achieved (which can be quite low), is the critical parameter in cell kill, and may even afford the attainment of selectivity. High-time chemotherapy has been discussed previously from the perspective of tumor cell kill (19, 20), but our results suggest that it may also apply to endothelial cell kill and inhibition of growth as well.

Long-term, continuous-like exposure of cells in culture to anticancer chemotherapeutic drugs has not been undertaken previously because the main objective of such experiments in the past has been to study the toxic effects of relatively high concentrations of such drugs on tumor cells. Therefore, brief exposure (*e.g.*, 1 h), followed by assessment of effects on tumor cell viability and clonogenicity, have been typical in such experiments. The resulting IC_{50} values of conventional chemotherapeutic agents usually depended on numerous factors such as exposure time, pharmaceutical preparation, drug metabolism, and the type of cancer cells treated. Because of such factors, especially the short exposure times, the published effective IC_{50} drug concentrations on human cells of drugs such as PTX (21), EpoB (21), and BMS-275183 (13) tend to be much higher than the results of our experiments. In contrast, we studied the effects of protracted drug exposure using very low concentrations of drug and tested the effects of such protocols not only on tumor cells but on normal fibroblasts and vascular endothelial cells. The rationale for doing so stems from the concept of using continuous low-dose metronomic/antiangiogenic chemotherapy regimens as a means of inducing antitumor effects *in vivo* secondary to an effect on the endothelial cells of a tumor's vasculature (4, 5). Results by Browder *et al.* (4) and Klement *et al.* (5) have shown this can cause long-term growth control of transplanted tumors with little host toxicity, and without the rapid development of drug resistance. Indeed, tumors selected for high levels of acquired resistance can be induced to respond by using metronomic/antiangiogenic chemotherapy regimens (4, 7), especially when they are combined with an antiangiogenic drug such as TNP-470 or anti-VEGF receptor-2 blocking antibodies (4, 7).

Our results are highly suggestive of an antiangiogenic window when low-dose chemotherapy is used, which becomes apparent after protracted exposure times (*e.g.*, 144 h) where drug-containing medium is replaced on a daily basis. There was a clear trend showing the effectiveness of low concentrations of drug that had no or little effect at low dose on tumor cells or normal fibroblasts (*e.g.*, 25–150 pM for PTX, BM-S275183, or EpoB), in contrast to microvascular endothelial cells. This was the case not only for inhibition of proliferation but also for the induction of apoptosis. Other functions such as cell migration and adhesion to extracellular matrix, which are all consid-

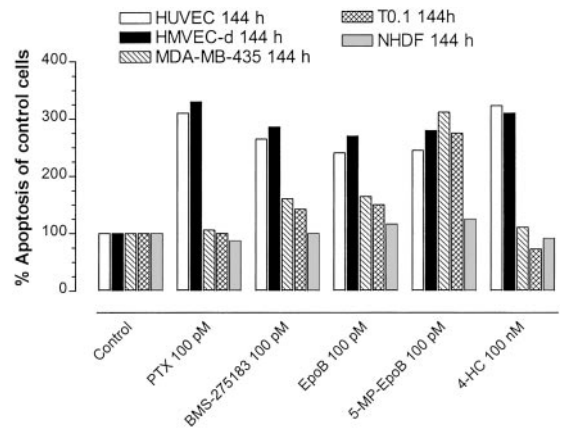

Fig. 4. Induction of cell apoptosis after prolonged (144 h) low-dose treatment with chemotherapeutic drugs on the HUVEC, HMVEC-d, MDA-MB-435, T0.1 and NHDF cell lines. Apoptosis was detected by a Cell Death ELISA kit, and the results were plotted as a percentage of the apoptosis of control cells (100%).

ered relevant to angiogenesis, are also similarly affected in a differential and selective manner (data not shown).[4]

Taken together, the results provide further evidence that some of the antitumor effects of anticancer chemotherapy drugs may be attributable to inhibition of tumor angiogenesis (1). Moreover, it may be possible to significantly lower drug concentrations with the aim of reducing overall host toxicity, but without sacrificing and even increasing antitumor efficacy, as emphasized by Browder *et al.* (4), Klement *et al.* (7), and Bello *et al.* (22). An additional benefit would be to delay acquired drug resistance (5), and even to treat tumors that are already resistant to the very drugs used for the low-dose chemotherapy (5, 7), given the relative genetic stability of normal host vascular endothelial cells, in contrast to tumor cells (23).

Whereas our experiments have dealt mainly with conventional or new cytotoxic chemotherapy drugs, the results may apply to other types of anticancer drugs as well. Indeed, we have found that BAL-9504, a geranylgeranyl-transferase inhibitor (24), had selective antiendothelial cell effects using protracted low-dose exposures.[4] We have been studying BAL-9504 in our analysis because there is evidence that similar drugs (*e.g.*, Ras farnesyltransferase inhibitors) may have direct endothelial cell effects and, hence, might inhibit angiogenesis through such effects (25) as well as by interfering with certain tumor cell functions relevant to angiogenesis, such as VEGF production (26).

Four other aspects of our experimental system, and results, need to be emphasized. First, the relative sensitivity of vascular endothelial cells to protracted low-dose chemotherapy may vary with the organ origin of endothelial cells. In addition, a potential weakness of our study is that we did not include types of normal cell that are ordinarily highly sensitive to chemotherapy *in vivo* (*e.g.*, gut mucosal epithelial cells, hair follicle cells, or bone marrow progenitors). It is possible that such cell types may show a high degree of sensitivity to protracted exposures of low concentrations of chemotherapeutic drugs, similar to endothelial cells. This is under investigation. Second, our previous *in vivo* results using regimens involving frequent or continuous administration of low-dose chemotherapy (5, 7, 12) have emphasized the critical need for combining such regimens with a second antiangiogenic agent, such as anti-VEGF receptor-2 blocking antibody (5, 7), a finding confirmed by others (22, 27–29). For example, the intrinsic elevated sensitivity of activated endothelial cells, to low-dose chemotherapy, compared with other cells, may not be expressed as a result of the presence of high local concentrations of endothelial cell-specific survival factors such as VEGF (30–33). Such combinations may be particularly effective in inducing higher levels of apoptosis of activated endothelial cells (7) coupled with the inhibition of cell proliferation, especially when used in a protracted manner. Third, an obvious question raised by our results is why vascular endothelial cells appear to be selectively sensitive to protracted exposure of low concentrations of chemotherapeutic drugs. It is possible that the inhibition of endothelial cell growth or induction of apoptosis may not be directly mediated by the chemotherapeutic drugs tested, but rather are secondary to an event induced by the drugs (*e.g.*, a change in expression of genes or proteins that mediate in some fashion the selective antiendothelial effects we observed). Fourth, it is interesting that some of the drugs we tested expressed potent antitumor cell activity as well as antiendothelial cell activity, when used at ultra-low concentrations in a protracted manner (*e.g.*, EpoB). Such an agent, if it functioned in a similar manner *in vivo*, may be particularly useful for metronomic chemotherapy protocols because it may affect both the tumor cell and endothelial compartments in a chronic manner, and do so in a way that avoids the acute toxicities normally associated with MTD-based, tumor cell-directed, chemotherapeutic regimens.

In summary, our results add to a small but important, and growing, body of literature implicating conventional (and new) chemotherapy drugs as antiangiogenics, a property that can be enhanced by protracted exposures to low doses of drug. Our results may also be directly relevant to *in vivo* therapy studies using other known, or potential, antiangiogenic agents such as IFNα and endostatin where these drugs appear to have greater antiangiogenic and antitumor effects when administered frequently or continuously at low doses (34–36).

[4] G. Bocci and R. S. Kerbel, unpublished observations.

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