Effects of Chemotherapeutic Drugs on Platelet and Metastatic Tumor Cell-Endothelial Cell Interactions as a Model for Assessing Vascular Endothelial Integrity

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ABSTRACT

An in vitro assay for examining the sublethal effects of chemotherapeutic agents on vascular endothelial integrity is described. Using vascular endothelial cell monolayers, the kinetics of binding of radiolabeled platelets or metastatic tumor cells were found bound only to the exposed areas of subendothelial matrix. Some drugs (bleomycin, 1,3-bis(2-chloroethyl)-1-nitrosourea, vincristine) induced rapid endothelial cell retraction and increased platelet and tumor cell binding to exposed subendothelial matrix, while one of the drugs tested (Adriamycin) caused delayed (1 to 3 days after a 2-hr drug treatment) endothelial cell retraction and increased cell binding. Of the drugs tested, only 5'-fluoro-2'-deoxyuridine which interferes with DNA replication failed to induce endothelial cell retraction and increased tumor cell and platelet binding. The results suggest that certain drug effects on the vascular endothelium can be assessed using the vascular endothelial cell monolayer model.

INTRODUCTION

The prevention of cancer deaths depends to a large degree on the successful eradication of highly malignant cells and interdiction of the metastatic process. Although successful treatment regimens have been developed for certain patients with particular cancers (6, 12), many patients succumb to metastatic disease at sites distant from their primary cancers (28). The formation of distant hematogeneous metastases requires that the blood-borne tumor cells implant within the microcirculation, usually by adhesion to endothelial cells in a blood vessel and to the underlying basal lamina matrix. Subsequently, the tumor cells must penetrate the cell and matrix layers of the basal lamina exposed, the attachment of metastatic blood vessel lumen to the basal lamina matrix surface (16, 24). If vascular endothelial cells are killed or damaged and subendothelial basal lamina exposed, the attachment of metastatic cells is enhanced (10). We have used the vascular endothelial cell monolayer adhesion assay to test the effects of various chemotherapeutic drugs on vascular endothelial cell integrity. By examining drug effects on the exposure of subendothelial matrix and attachment of radiolabeled B16 melanoma cells or platelets in vitro, we have sought to determine which drugs might damage endothelium and increase tumor cell interactions with the subendothelial basal lamina.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. A murine B16 melanoma subline (B16-F1) was obtained from Dr. I. J. Fidler (then at the Frederick Cancer Research Facility, Frederick, MD), and was grown in a 1:1 mixture of DME and F-12 (Grand Island Biological Co., Grand Island, NY) containing 5% FBS (HyClone Laboratories, Logan, UT). The B16 cells were harvested by treatment for 10 min with 2 mm EDTA in calcium-, magnesium-free DPBS. After suspension into single cells and washing by brief centrifugation and resuspension in DME:F-12 with serum, cell viabilities were determined by trypan blue dye exclusion. Cloned cell bovine aortic and corneal endothelial cells were obtained from Dr. D. Gospodarowicz (University of California, San Francisco, CA), or they were established in our laboratory. Endothelial cells were grown on gelatin-coated 16-mm 24-well Costar culture plates in a 1:1 mixture of DME:F-12 medium containing 10% FBS. All assays used cell maintained in culture for no more than 10 passages from frozen stocks. Fibroblast growth factor (100 to 500 ng/ml) was added every other day as described (3).

Drugs. ADM (Doxorubicin) was obtained from Adria Laboratories (Columbus, OH); BCNU (Carmustine) and BL (Bleomycin) were products of Bristol Laboratories (Syracuse, NY). BCNU was dissolved in dimethyl sulfoxide immediately before use. VCR (Oncovin) was produced by Eli Lilly and Co. (Indianapolis, IN), and Fluorouridine (Floxuridine) was obtained from Roche Laboratories (Nutley, NJ).

Adhesion Assays. B16 melanoma cells were radiolabeled as described previously (14, 21) with 0.25 mCi of Na125I or 0.25 mCi of Na51CrO4 (carrier-free; New England Nuclear, Boston, MA) per 100-mm culture dish for 3 hr in DME:F-12 containing 5% FBS. After labeling, the B16 cells were suspended in the same solution at a concentration of 4 x 10^5 cells/ml. Rabbit platelets were obtained and labeled as follows. PRP was prepared from 20 ml of citrated blood (0.38% sodium citrate) by centrifugation at 200 g for 15 min at 4° (10, 24). This may explain why metastatic cells move from the blood vessel lumen to the basal lamina matrix surface (16, 24).

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The abbreviations used are: DME, Dulbecco-modified minimal essential medium; ADM, Adriamycin; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CARMA, Carmustine; BLM, bleomycin sulfate (Blenoxane); BSA, bovine serum albumin; DPBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; F1UdR, 5'-fluoro-2'-deoxyuridine (Floxuridine); PRP, platelet-rich plasma; VCR, vincristine sulfate (Oncovin).
PRP was layered over 1 ml of a BSA (40 to 45%; Fraction IV; Sigma Chemical, St. Louis, MO) solution, and the 2-phase solution was centrifuged at 1200 x g for 10 min at 21 °C (31). The platelets were harvested from the interface of the BSA solution and were resuspended in calcium-, magnesium-free DPBS containing 0.38% sodium citrate solution. The platelets were recentrifuged as before and were removed and suspended in calcium-, magnesium-free DPBS containing 8 units per ml of heparin (31).

The kinetics of adhesion of radiolabeled B16 melanoma cells or rabbit platelets to untreated and drug-treated endothelial cell monolayers in Costar 24-well plates were determined at 37 °C as described previously (14, 21). The endothelial cell monolayers were incubated for 2 hr with varying concentrations of drug and washed 3 times with conditioned media prior to the adhesion assay. In control experiments, the endothelial cells were incubated for similar times in drug-free media before the adhesion assays were conducted. For the adhesion assays, platelets were used at a concentration of 0.8 to 1 x 10⁸/ml including 2 units per ml of heparin, or B16 cells at a concentration of 4 x 10⁶ cells/ml in DME plus 12 mM N-2-hydroxymethylpiperazine-N'-2-ethanesulfonic acid and 1% BSA.

Optical and Electron Microscopy. Various untreated and drug-treated endothelial cell monolayers were unfixed or fixed in 10% formalin and examined by phase microscopy. For scanning electron microscopy, untreated and drug-treated endothelial cell monolayers were carefully washed with DPBS at 37 °C and fixed at this temperature with 1% buffered glutaraldehyde:0.125 M sodium cacodylate buffer, pH 7.3 for 1 hr at 37 °C. The endothelial cell monolayers were rinsed in 0.125 M sodium cacodylate buffer for three 3-min periods and were postfixed in 2% OsO₄ in the same cacodylate buffer for 30 min at 22 °C. After 3 more rinses in cacodylate buffer, the cell monolayers were dehydrated in a graded series of ethanol, transferred to Freon 113, and critical-point dried in a Ladd critical-point dryer (Ladd Research Laboratories, Burlington, VT). The endothelial cell monolayers were coated with 50 to 100 A gold and platinum in a Hummer VI (Technics, Springfield, VA) and examined in a Hitachi Model S520 scanning electron microscope (16, 17).

RESULTS

Drug Effects on Tumor Cell and Platelet Binding to Endothelial Cell Monolayers. Chemotherapeutic drugs were examined for their effects on endothelial cells using the endothelial cell monolayer assay to monitor drug-induced changes in endothelial cells. After a 2-hr treatment with low concentrations of BLM (0.0008 units/ml) (Chart 1A), VCR (80 ng/ml) (Chart 1B) or BCNU (10 µg/ml) (Chart 1C) endothelial cells still excluded vital dyes, but the kinetics of adhesion of B16 melanoma cells to the drug-treated endothelial cell monolayers were significantly modified. At higher concentrations of these agents (BLM, 0.01 units/ml; VCR, 1 µg/ml; or BCNU, 40 µg/ml), endothelial cells became rounded and/or detached from their matrix. These cells were considered nonviable, since they failed to reattach, spread, and proliferate even in drug-free media. The drug exposure times required for maximal enhancement of melanoma cell attachment in a 30-min adhesion assay varied: 6 hr for BLM, 0.008 units/ml; 30 min for VCR, 800 ng/ml; and 15 to 20 min for BCNU, 10 µg/ml. Dose-response data for different drug concentrations using a 2-hr drug exposures and 75% B16 cell attachment at 30 min were: BLM, 0.0008 units/ml; VCR, 80 ng/ml; and BCNU, 5 µg/ml. Certain agents such as ADM (Chart 2A) did not cause changes in the kinetics of melanoma cell adhesion to the drug-treated endothelial cells when assayed immediately after the 2-hr drug incubation; however, when these same endothelial cell monolayers were placed in fresh medium plus FBS and were assayed 1 to 3 days after the drug incubation, dramatic differences in the rates of B16 cell adhesion to the endothelial cell monolayers were observed. Concentrations as low as 0.3 µg per ml of ADM for 2 hr resulted in different kinetics of melanoma cell adhesion at 3 days postincubation (Chart 2B).

Some of the drugs tested for their effects on endothelial cells did not cause a change in the kinetics of B16 melanoma cell adhesion to the endothelial cell monolayers. For example, concentrations of FdUrd up to 10 µg/ml for 2 hr had no effect on B16 cell adhesion to endothelial cell monolayers if assayed immediately after (Chart 3A) or 3 days after (Chart 3B) drug treatment of the endothelial cells.

The results obtained by measuring radio-labeled B16 melanoma cell adherence to control and drug-treated endothelial cell monolayers were similar to those obtained with purified radio-labeled platelets. Platelets bind poorly to intact, confluent endothelial cell monolayers, but treatment of endothelial cells with low concentrations of BCNU, VCR, or BLM resulted in increased platelet binding to the drug-treated endothelial cell monolayers (Chart 4). FdUrd (up to 10 µg/ml for 2 hr) did not result in increased kinetics of platelet binding (Chart 4).
DRUG-INDUCED ENDOTHELIAL CHANGES

Chart 2. Kinetics of radiolabeled B16 melanoma cell adhesion to untreated and ADM-treated endothelial cell monolayers assayed immediately after a 2-hr drug treatment (A), or 3 days after a 2-hr drug treatment (B). Data points are mean values; bars, S.E.

Chart 3. Legend is the same as in Chart 2 except that untreated and FdUrd-treated endothelial cell monolayers were used.

Ultrastructure of Drug-treated Endothelial Cell Monolayers.
Since exposure of the subendothelial basal lamina-like matrix modifies the kinetics of adhesion of melanoma cells to endothelial cell monolayers (16, 21), we examined the integrity of such monolayers before and after drug treatment (17). Control endothelial cell monolayers were intact and confluent and rarely showed areas of exposed subendothelial matrix (Fig. 1A). However, treatment of endothelial cell monolayers with drugs using the same concentrations and times of chemotherapeutic drugs used for the adhesion assays resulted in dose-dependent endothelial cell retraction and exposure of subendothelial matrix (Fig. 1B) except for the FdUrd-treated samples. Adhesion of B16 melanoma cells to drug-treated endothelial cell monolayers resulted in widespread tumor cell attachment to exposed subendothelial matrix (Fig. 1, C and D). In the case of BCNU treatment, melanoma cells also bound to endothelial cells (Fig. 1D).

The addition of platelets to control endothelial cell monolayers rarely resulted in platelet binding to the endothelial cells, although a few platelets will occasionally bind to the parameters of the endothelial cell monolayers. After treatment with chemotherapeutic drugs that modify platelet binding to the endothelial cell monolayers, such as BCNU, VCR, or BLM, retraction and exposure of the subendothelial matrix occurred, and the platelets bound to the exposed matrix (Fig. 2, B to D). The exception was endothelial cell monolayers treated with FdUrd where endothelial cell retraction and platelet binding did not occur (Fig. 2A).

DISCUSSION

Drug toxicity is a serious side effect of cancer chemotherapy and can limit the usefulness of most of the commonly used drugs. In some cases, toxicity can be directly traced to vascular endothelial cell damage. For example, BLM, a complex of watersoluble peptide antibiotics extracted from Streptomyces verticillus (29), has been shown to be an effective agent against number of cancers, such as malignant lymphomas (5, 26), testicular cancers (25), and carcinomas of the head and neck (13). However, at clinically effective doses, systemic administration of BLM can cause lung and skin lesions, resulting eventually in interstitial fibrosis (4, 32). Evidence suggests that the initial injury can be attributed to BLM damage of the endothelial cells lining pulmonary and cutaneous arteries and veins (1, 7). This is thought to be the cause of the observed increase in cutaneous and pulmonary vascular permeability (9) and the arrest and infiltration of perivascular spaces by lymphocytes and plasma cells (1).

The damaging effects of chemotherapeutic drugs on vascular endothelium have been assessed, for the most part, by in vivo functional and morphological studies. In the case of BLM, endothelial cell damage has been monitored by single-pass removal of [14C]-5-hydroxytryptamine and [3H]norepinephrine after i.v. injection, by measurement of serum angiotensin converting en-
zyme activity (7), and by conventional histological and electronmicroscopic examination (1, 2).

Using cultured vascular endothelial cells, we sought to develop a rapid, reproducible in vitro technique for assessing drug-induced vascular damage. To be useful in drug screening, large numbers of test samples at multiple concentrations and combinations would have to be assayed rapidly. Therefore, in our assay system, endothelial cell monolayers were established in 16-mm 24-well plates, allowing large numbers of drugs and other agents to be assayed quickly in parallel. As an indicator of endothelial cell damage, we chose sublethal criteria that result in exposure of subendothelial basal lamina-like matrix and increased binding of platelets and tumor cells to the exposed matrix. These effects could also be monitored by phase and scanning electron microscopy.

In examining the effects of various chemotherapeutic drugs on vascular endothelial cell monolayer integrity at clinically relevant concentrations, we found that the drugs could be placed into 3 basic groups: drugs that cause immediate effects on vascular endothelial cell integrity, such as BCNU, BLM, and VCR; drugs that cause delayed effects on vascular endothelial cell monolayer integrity, such as ADM; and drugs that have no apparent effect on vascular endothelial cells, such as FdUrd. This latter drug is thought to act exclusively by interfering with DNA replication. Since confluent monolayers of vascular endothelial cells, as well as endothelial cells lining blood vessel walls, do not normally contain dividing cells, the integrity of the endothelium may not be affected by such replication-specific drugs. It follows that the other drugs tested can cause effects in quiescent, normal endothelial cells. We did not examine drugs that required activation, such as cyclophosphamide, although use of a microscope preparation along with such drugs could allow their testing in our assay. For the most part, our studies were conducted with BAE cells. Although these vascular endothelial cells are easy to grow and maintain, their dose-response to the various drugs is not equivalent to that of other cells such as bovine corneal endothelial cells (less sensitive to BLM and BCNU) or human microvascular endothelial cells. We are currently examining a number of experimental drugs and directly comparing them in this assay to their structural analogues, as well as to the drugs used in the studies presented here.

Our results suggest that drug-induced vascular damage and exposure of subendothelial basal lamina could result in increased blood-borne implantation of circulating malignant cells and enhanced metastasis formation. This has been observed experimentally in animals that were pretreated with chemotherapeutic agents before administration of tumor cells i.v. In these experiments, higher numbers of pulmonary tumor nodules were found in drug-treated animals than in untreated animals (11, 18, 30). This could present a problem, clinically, if drug-resistant variants exist in a malignant cell population. The probability that such cells might escape cytotoxic therapy and succeed in completing all of the steps required for blood-borne metastatic colonization could be enhanced under circumstances where vascular integrity is compromised due to drug effects on vascular endothelial cells.

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Fig. 1. Scanning electron-microscopic examination of untreated and drug-treated endothelial cell monolayers fixed immediately after a 2-hr drug treatment. A, untreated control; B, BCNU, 5 μg/ml; C, BCNU, 5 μg/ml, for 2 hr, then B16 cells were added; D, BCNU, 12 μg/ml, for 2 hr then B16 cells were added. Bars = 5 μm; T, tumor cell.
Fig. 2. Legend is the same as in Fig. 1 except that radiolabeled platelets were added after a 2-hr drug treatment. A, FdUrd, 7 μg/ml; B, VCR, 40 ng/ml; C, BCNU, 2.5 μg/ml; D, BLM, 0.0008 unit/ml. Bars = 5 μm; P, platelet.
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