Studies on the Mechanisms Responsible for Inhibition of Experimental Metastasis of B16-F10 Murine Melanoma by Pentoxifylline

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Abstract
Pentoxifylline (PTX), a methylxanthine derivative widely used as a hemorheological agent in the treatment of peripheral vascular disease, was studied to unveil the mechanisms responsible for its inhibitory action on B16-F10 experimental metastasis. In vitro pretreatment of B16-F10 cells with noncytotoxic concentrations of PTX significantly inhibited their adhesion to reconstituted basement membrane Matrigel® and type IV collagen as well as the relative activity of secreted 92 kD metalloprotease. However, PTX pretreatment of B16-F10 cells did not affect their in vitro invasiveness. Heterotypic organ adhesion assays carried out with B16-F10 cells and suspended organ tissues demonstrated that pretreatment with noncytotoxic concentrations of PTX of both, tumor cells or lung tissue, brought about a dose-dependent inhibition of melanoma cell adhesion to lung. Immunohistochemical studies using antibodies against CD31 adhesion molecule (PECAM-1) revealed that B16-F10 cells adhere to lung endothelial cells. Our results suggest that PTX may exert its inhibitory effect on tumor lodgment, and as a consequence of that on experimental metastases, through an inhibitory action on cell adhesion molecules.

The metastatic dissemination of malignant melanoma is crucial to determine the prognosis of patients with that disease. Treatment of early lesions by accurate surgical excision can result in near 100% cures. On the contrary, there is currently no effective chemotherapeutic agent or combination that has invariably yielded regressions in metastatic melanomas [8, 24]. Therefore, the finding of new therapies in these cases is essential to control this highly lethal disease.

Pentoxifylline (PTX), a methylxanthine derivative, is used as a hemorheological agent in the treatment of peripheral vascular disease [25]. Due to some of its characteristics, such as inhibitor of platelet aggregation and thrombus formation [1], cytoskeleton depolymerizer [22], tumor necrosis factor (TNF) suppressor [17], and cell membrane fluidity modifier [19], among others, it has lately been the focus of some studies in experimental cancer. PTX has been reported to enhance chemo- and radiosensitivity in some cancer cells [7, 10] and to affect tumor
growth, angiogenesis and metastasis in certain models [2, 3, 9].

Recently, one of us has demonstrated the inhibitory effect of PTX on the lung colony-forming ability of one cell subline derived from B16-F1 melanoma, that was selected in vivo for enhanced lung metastatic ability [14]. In an extension of this work, we investigate here the effect of PTX on B16-F10 cell in vitro invasiveness, adhesion to basement membranes, motility, metalloproteinase (MMP) and urokinase-type plasminogen activator (uPA) secretion and activity, in order to identify possible mechanisms of PTX antimetastatic action. Studies aimed at analyzing the effect of the methylxanthine derivative on the interactions of B16-F10 cells with lung, organ preferentially colonized by the melanoma cells, were also carried out. The results obtained suggest that PTX may exert its antimetastatic effect by inhibiting tumor cell lodgment at the metastatic target organ.

Materials and Methods

Cell Lines

B16-F10, a highly metastatic lung-selected subline derived from the C57BL/6 B16 murine melanoma [11], was originally supplied by Dr. Daniel Hansburg (Fox Chase Cancer Center, Philadelphia, Pa., USA). The cell line was regularly grown in MEM Eagle medium with nonessential amino acids (Sigma Chemical Co., St. Louis, Mo., USA) supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum, FBS (GEN, Buenos Aires, Argentina), MEM vitamin solution, L-glutamine, sodium pyruvate and antibiotics. The cell line was maintained at 37°C in a 5% CO2 humidified atmosphere.

Chemosensitivity Assay

B16-F10 cells in exponential growth were harvested, resuspended in MEM without phenol red supplemented with 10% FBS, and seeded in 96-well tissue culture plates at a concentration of 5 x 10^4 cells/100 μl per well. The cells were allowed to attach to the surface for 24 h and then exposed for 2 h to different doses of PTX (Genitum DL®, Laboratorios LANDO, Buenos Aires, Argentina) prepared in complete medium or absence of PTX (untreated control). The selected doses of PTX were 0.36, 3.6, 36, 90 and 180 μM at final concentration, respectively. The cells were washed with PBS and then allowed to grow in drug-free MEM medium containing FBS for 24 h. The colorimetric assay Cell Titer 96®Aqueous (Promega, Madison, Wisc., USA) using the tetrazolium inert salt MTS and phenazine methosulphate (PMS) was used to determine formazan, reduced form of the tetrazolium compound due to mitochondrial dehydrogenases, in viable cells. Briefly, 20 μl of MTS/PMS were added per well and plates were incubated for 4 h at 37°C. The optical density was measured in an ELISA plate reader at 490 nm wavelength. Every single dose of PTX was assayed in triplicate. The inhibitory concentration IC_{50} of PTX was determined as the drug concentration that decreased 50% the optical density of the control untreated cells.

Chemoinvasion Assay

Tumor cell invasion assay was carried out in 12-μm-pore Transwell® cell culture chamber inserts (Corning Costar, Cambridge, Mass., USA) as previously described [5]. Briefly, the upper surface of the polycarbonate membrane was coated with 50 μg/100 μl of cold reconstituted basement membrane Matrigel® (Collaborative Biomedical Products, Bedford, Md., USA). B16-F10 cells in semiconfluency were pretreated for 2 h with noncytotoxic doses of PTX (1.8 and 3.6 mM) or MEM medium with 0.1% bovine serum albumin BSA (control), and then seeded at a cell concentration of 1 x 10^4/100 μl in the upper chamber. The lower compartment was filled with conditioned medium from NIH-3T3 cells used as chemoattractant. Following 18-20 h incubation at 37°C in a humidified 5% CO2 atmosphere, the content of the upper compartment was gently aspirated and the lower chamber and undersurface of the polycarbonate membrane treated with 0.25% trypsin/EDTA. The cell suspension, which represents cells capable of moving through the filter in response to the chemoattractant, was centrifuged at 1,000 rpm for 10 min and the resultant pellet resuspended in 20 μl of PBS to count cells in a Neubauer chamber. All the assays were carried out in quadruplicate.

Chemotaxis or Motility Assay

The chemotaxis assay was carried out in a similar way as for chemoinvasion assay except that the polycarbonate membrane was pre-coated with cold collagen type IV (Sigma) at 5 μg/100 μl.

Cell Adhesion Assay

Semi-confluent B16-F10 cells were pretreated with 0, 1.8 and 3.6 mM PTX in complete culture medium for 2 h at 37°C. The cells were washed twice with PBS and harvested using 0.25% trypsin/EDTA. The cell suspensions obtained were adjusted to 5 x 10^5 cells/ml in MEM without phenol red containing 0.1% BSA and 100 μl seeded per well in 96 microtitre plates precoated with 40 μl of Matrigel (50 μg/100 μl) or 40 μl of type IV collagen (5 μg/100 μl). The plates were incubated for 30, 60 and 90 min at 37°C. The walls were aspirated at the indicated times and washed twice with PBS to remove nonadherent cells. Adhesion was quantified by the MTS colorimetric assay at 490 nm as explained above, and expressed as a percentage of the respective total number of cells (wells containing adherent and nonadherent cells). All assays were performed in quadruplicate.

Zymography

Cell proteinases secreted by untreated as well as by PTX-treated (1.8 and 3.6 mM for 2 h) B16-F10 cells, were obtained from MEM medium containing 0.1% BSA to which semiconfluent cell cultures were exposed for 48 h at 37°C. Collected conditioned media (CM) were diluted to normalize the concentration of enzymes secreted into the medium with reference to the flask containing the lower number of cells. CM were concentrated 14 times with Ultrafree®-CL centrifugal filters for sample concentration (Millipore Corporation, Bedford, Mass., USA) with a 30-kD molecular weight cut-off. The concentrated CM samples were stored frozen (-20°C) until use. Gelatinase activity was detected by zymography after SDS-PAGE 7.5% using gels containing 1 mg/ml gelatin [6]. Gels were stained with 0.25% Coomasie brilliant blue R-250 (Sigma) and destained in 30% methanol and 10% glacial acetic acid in H2O. Gelatinolytic activity in CM was evidenced as transparent bands against the dark-blue background due to stained gelatin. The intensity of bands was quantified.