Effects of the pyrimido-pyrimidine derivative RX-RA 85 on metastatic tumor cell–vascular endothelial cell interactions

ROSEMARIE B. LICHTNER and GARTH L. NICOLSON†
Department of Tumor Biology, The University of Texas M.D. Anderson Hospital and Tumor Institute at Houston, 1515 Holcombe Boulevard, Houston, TX 77030, U.S.A.

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An important step in the metastatic process is the interaction of blood-borne malignant cells with the vascular endothelium. Among the agents that may interfere with this process are pyrimido-pyrimidines, such as RX-RA 85, developed originally as an antiplatelet agent. Using an endothelial cell monolayer attachment assay we have investigated the effects of RX-RA 85 on tumor cell and endothelial cell properties. Exposure of bovine aortic endothelial cells for 3 h to >4 μg/ml RX-RA 85 produced toxic effects, resulting in vacuole formation, retraction and finally rounding up of the cells. Endothelial cells derived from different sources behaved dissimilarly; human brain, human meninges, mouse brain, mouse lung and rat lung endothelial cells were less sensitive to drug treatment than bovine aortic endothelial cells. RX-RA 85 treatment of bovine aortic endothelial cells increased B16-F1 melanoma cell adhesion. When B16-F1 cells were exposed to 4–8 μg/ml RX-RA 85, increased adhesion to the subendothelial matrix occurred, whereas exposure to higher drug concentrations (8–16 μg/ml RX-RA 85) decreased adhesion. Indirect immunofluorescence staining of cytoskeletal structures in B16-F1 cells adhering to and spreading on matrix revealed that the differential effects of RX-RA 85 on the organization of microtubules and microfilaments might explain the dose-dependent differences in adhesion kinetics.

Introduction

Metastasis formation is a complex phenomenon involving several sequential steps [13, 29, 30, 32, 33]. Important in this process is the transport of tumor cells in the circulation where they can interact with each other, host platelets, lymphocytes, and the vascular endothelium [6, 7, 29, 30]. Tumor cells attach relatively slowly to the apical surfaces of endothelial cells, though they generally attach more rapidly and strongly to subendothelial extracellular matrix [20, 31]. After their implantation in a small blood vessel, tumor cells are often found adherent to regions of exposed subendothelial matrix [5, 39].

The adhesion of tumor cells to endothelial cells and their extracellular matrix in vitro and the formation of experimental metastases in vivo have been inhibited by substances that affect cytoskeletal structures, such as cytochalasin B, colchicine [12], and local anesthetics [35]. Another class of drugs that interferes with the organization of the tumor cell cytoskeleton [25, 26] and reduces metastases in some animal tumor systems [23, 24] are the pyrimido-pyrimidines, which were originally developed as antiplatelet drugs. The antimetastatic effects of the pyrimido-pyrimidines have been inconsistent [23, 24]. These drugs can inhibit in a dose-dependent manner the adherence of circulating tumor cells to the endothelium of

† To whom requests for reprints should be addressed.
mesenteric blood vessels [8, 9]. They are also inhibitors of platelet (R. Weisenberger, unpublished results) and tumor cell [26; R. Zimmermann, unpublished results] phosphodiesterases, and they can inhibit nucleoside uptake [18, 26, 28], stimulate prostacyclin (PGI\textsubscript{2}) biosynthesis [3], and enhance the number of plaque-forming cells in the spleens of low-dose immunized mice [22].

To study the effects of pyrimido-pyrimidines on tumor cell adhesive properties we used the tumor cell-endothelial cell monolayer adhesion system [29, 34]. For this investigation we chose the extremely potent pyrimido-pyrimidine derivative RX-RA 85 [23, 24], which interferes with the growth and cytoskeletal organization of tumor cells at very low concentrations [25].

**Materials and methods**

**Cell lines and culture conditions**

Murine B16-F1 melanoma was obtained from Dr I. J. Fidler and was grown in a 1:1 mixture of Dulbecco’s-modified Eagle’s (DME) and F12 medium (Grand Island Biological Company, Grand Island, NY, U.S.A.) containing 5 per cent fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, U.S.A.) [16]. Subconfluent B16 cell cultures were harvested by treatment for 10 min with 2 mM EDTA in calcium–magnesium-free Dulbecco’s phosphate-buffered saline (PBS). Cloned bovine aortic endothelial (BAE) cells were obtained from Dr D. Gospodarowicz (University of California, San Francisco, CA, U.S.A.) or they were established in this laboratory. Rat lung, mouse lung, cloned mouse brain, cloned human brain, and human meninges endothelial cells were established in this laboratory by P. Belloni (Belloni and Nicolson, unpublished results). Endothelial cells were grown on gelatin-coated 16 mm 24-well Costar culture plates in a 1:1 mixture of DME : F12 medium containing 10 per cent FBS, with the exception of BAE cells which were also grown in the presence of 10 per cent horse serum derived from platelet-poor plasma (Hyclone). The medium did not contain antibiotics, and 50 μg/ml endothelial cell growth factor (ECGF) (Biomedical Technologies, Inc., Cambridge, MA, U.S.A.) was added every other day as described previously [2]. Cultures were last fed 3 days before the experiment. Cells were harvested using 0·125 per cent trypsin in 2 mM EDTA in DPBS free of calcium and magnesium (PBS). All assays used cells from frozen stocks maintained for no more than 10 passages (except the rat lung endothelial cells).

**Drug**

The pyrimido-pyrimidine derivative RX-RA 85 has been described by the manufacturer as a potent inhibitor of platelet (H. Weisenber, unpublished results) and tumor cell (R. Zimmermann, unpublished results) phosphodiesterases. RX-RA 85 was a gift from Dr Karl Thomae GmbH, Biberach, F.R. Germany, and was solubilized in hydrochloric acid and diluted in PBS as described previously [23, 24]. The drug solution was made up fresh daily.

**Toxicity studies**

Subconfluent B16-F1 cells were harvested by EDTA treatment, washed once in adhesion medium (DME containing 12 mM HEPES, 1 per cent bovine serum albumin BSA, pH 7·2), and then incubated (6 × 10⁵ cells) in 15 ml of adhesion medium plus the indicated concentrations of RX-RA 85 at 37°C under shear forces.