The effect of butyric acid and retinoic acid on invasion and experimental metastasis of murine melanoma cells

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The effect of butyric acid (BA) and all trans-retinoic acid (RA) on murine melanoma cells was investigated in vitro and in vivo. The in vitro assays included 3H-IdU incorporation, adhesion, migration and invasion experiments. Butyric acid decreased 3H-IdU cellular incorporation within 24h and increased adhesion as measured by trypsin release of 3H-IdU labelled cells from either polycarbonate (p.c.) or Matrigel-coated p.c. membranes. Migration and invasion rates after 72 h were quantified by scanning electron microscopy (SEM). The invasion barrier consisted of Matrigel-coated p.c. membranes. Butyric acid significantly enhanced migration and invasion of B16a cells, while RA significantly decreased migration and invasion of B16a and K-1735 cells. Subcutaneous administration of either BA or RA pellets significantly decreased the number of lung nodules in the experimental metastatic assay. The experimental metastatic assay is defined as a tail vein inoculation protocol followed by subsequent lung evaluation.

Introduction

The role of differentiating agents in the treatment of neoplasms and metastases is becoming increasingly important. Since differentiating agents may be less host-toxic and less mutagenic than chemotherapy, they may be used between rounds of chemotherapy to help prevent regrowth and diversification of remaining tumor cells [24]. One such agent, 13-cis-RA (topically applied) has produced partial responses in patients with melanoma [18].

In vitro pretreatment of tumor cells with differentiation agents such as BA [36], dimethyl sulfoxide (DMSO) [35], and N-methylformamide (NMF) [39] has increased in vivo experimental metastasis. In addition, in vitro pretreatment with both RA and dibutyl cyclic AMP together has changed the pattern of organ metastasis of F9 embryonal carcinoma cells [37].

Few studies have investigated the in vivo effect of differentiation agents on metastasis without drug pretreatment. Experimental evidence indicates that both the route and timing of the administration of differentiation agents is critical. N-methylformamide, when given prior to tail vein injection of tumor cells, increased experimental metastases, while NMF given after tail vein injection decreased experimental metastases [39]. Dietary retinoids given to athymic mice decreased the number of spontaneous metastases produced by untreated hamster melanoma HMI-FS cells [31].

A positive correlation has been shown between the in vitro reconstituted basement membrane (RBM) invasion assay and in vivo metastasis [1, 28]. The RBM

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model consists of a porous Transwell p.c. membrane (Costar Co., Cambridge, MA) that is coated with a reconstituted basement membrane (Matrigel, Collaborative Res., Bedford, MA). Matrigel is derived from the murine Engelbreth–Holm–Swar (EHS) sarcoma cell line and consists of laminin, collagen type IV, heparan sulfate proteoglycan and entactin [13]. During invasion, tumor cells locally degrade the Matrigel and migrate through the porous p.c. membrane. The cells can then be quantified on the underside of the p.c. membrane by light microscopy [1, 12], radioactive labelling [28] or scanning electron microscopy.

The RBM model has been used to test the effect of various drugs on the invasion process. Drugs tested included cytoskeletal agents such as colchicine [44] and estramustine [43]. The two drugs chosen for this study, RA and BA, affect the cytoskeleton in some tumor cell lines [2, 15, 23, 30]. We have previously reported the effect of these two agents on the migration of murine B16a cells [21]. The short-term effects of BA on the invasion process in the human amnion basement membrane (HABM) assay have been reported [38]. Pretreatment for 24 h with BA enhanced the invasion of M5076 sarcoma cells [38]. Likewise, pretreatment of murine B16F10 melanoma cells for 24 h with RA increased invasion through the amnion. However, pretreatment with RA for 48–72 h decreased invasion [46]. Retinoic acid has been shown to inhibit invasion of tumor cells in the RBM assay without affecting cellular proliferation [22].

In our study, both in vitro and in vivo protocols were used to study invasion and metastasis. For the in vitro migration and invasion studies, B16a and K-1735 melanoma cells were treated with BA or RA for 3–6 days. For the in vivo study, syngeneic female C57BL6 mice were primed with all trans-RA or BA by subcutaneous implantation of drug pellets 1 day prior to tail vein injection of B16a cells.

**Materials and methods**

*Cell culture*

Murine B16a and K-1735 melanoma cells were obtained from the DCT Tumor Repository, NCFS-Frederick Cancer Research Facility (Frederick, MD). Murine NIH-3T3 cells were obtained from American Type Tissue Collection (Rockville, MD). Cells were cultured in Eagle's minimum essential medium (MEM) (Gibco, Grand Island, NY) containing Hank's salts and l-glutamine supplemented with sodium pyruvate (Sigma, St Louis, MO), 10 per cent heat-inactivated fetal calf serum (FCS) (Gibco) or 10 per cent NuSerum (Collaborative Res., Cambridge, MA), 1 per cent penicillin G-streptomycin-amphotericin B (Fungizone) (Gibco), MEM non-essential amino acids (Gibco), sodium bicarbonate (Sigma), and Hepes (Sigma) (final pH 7.3). Cells were cultured in a humidified incubator (37°C, 5 per cent CO₂) and were refed every 3 days. Cells were subcultured with trypsin/EDTA (Sigma) during log phase growth.

B16a cells were also obtained from metastatic lung nodules or subcutaneous tumors according to the following protocol. The cells, originally obtained from the repository, were grown to preconfluence in T-75 flasks, trypsinized, and suspended in PBS. Four female C57BL6 mice received an injection of 0.2 ml of PBS-tumor cell suspension (500,000 cells/ml) either in a subcutaneous site or in the lateral tail vein. Mice were killed after 21 days. Subcutaneous or lung tumors were removed and minced in MEM containing FCS. After 24 h of incubation, unattached cells and