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Suberoylanilide hydroxamic acid limits migration and invasion of glioma cells in two and three dimensional culture

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ABSTRACT

High grade gliomas are aggressive cancers that are not well addressed by current chemotherapies, in large measure because these drugs do not curtail the diffuse invasion of glioma cells into brain tissue surrounding the tumor. Here, we investigate the effects of suberoylanilide hydroxamic acid (SAHA) on glioma cells in 2D and 3D in vitro assays, as SAHA has previously been shown to significantly increase apoptosis, decrease proliferation, and interfere with migration in other cell lines. We find that SAHA has significant independent effects on proliferation, migration, and invasion. These effects are seen in both 2D and 3D culture. In 3D culture, with glioma spheroids embedded in collagen I matrices, SAHA independently limits both glioma invasion and the reorganization of the tumor surroundings that usually proceeds such invasion. The decreased matrix reorganization and invasion is not accompanied by decreased production or activity of matrix-metalloproteases but instead may be related to increased cell–cell adhesion.

1. Introduction

High grade gliomas are devastating brain tumors that are nearly uniformly fatal despite decades of intensive pre-clinical and clinical research. Although gliomas rarely metastasize outside of the central nervous system, their ability to spread within the brain limits the efficacy of all current treatment strategies [1–8]. Developing effective anti-invasive therapies to be used in combination with other surgical, chemotherapeutic, and radiative approaches is necessary to significantly change the prognosis of patients with these cancers. As a result, many studies have focused on identifying and targeting the cellular mechanisms that underlie glioma invasiveness. Matrix metalloproteases (MMPs), which degrade extracellular matrix components, have long been considered critical elements in tumor invasion and metastasis. In particular, studies have demonstrated increased expression of the gelatinases MMP-2 and MMP-9 and the membrane-anchored MMP-14 (or MT1-MMP) in and around high grade gliomas [9–16]. While such studies suggest MMP inhibition may be a critical tool in limiting glioma invasion, results of clinical trials of synthetic metalloprotease inhibitors thus far have been disappointing, and the search for effective anti-invasive treatments for high grade gliomas continues [17].

Recently, suberoylanilide hydroxamic acid (SAHA or vorinostat), a histone deacetylase inhibitor (HDACi), has been identified as a very promising anti-cancer drug for its ability to arrest cell growth and induce apoptosis of cancer cells while sparing normal cells in a variety of cell lines [18,19]. SAHA has been the focus of several studies against glioma in particular [20–24]. Administration of SAHA to mouse, rat, and human glioma cell lines in vitro limited cell proliferation and up-regulated pro-apoptotic and anti-proliferative genes including $p^{21/WAF}$ and $p^{27/KIP1}$ [20,22]. In mouse models, SAHA was shown to cross the blood–brain barrier, limit tumor volumetric growth, and increase survival time [20–22].

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While studies of the effects of SAHA on gliomas thus far have focused on SAHA's ability to halt cell growth and induce apoptosis, other studies suggest SAHA may be an effective anti-invasive compound as well. SAHA decreased tumor necrosis factor driven invasion through Matrigel coated Transwell filters in lung adenocarcinoma cells [25]. Other HDAC inhibitors have also been shown to limit invasion. Trichostatin A (TSA) and tubacin limited transformed fibroblast cell invasion through coated Transwell filters [26,27]. TSA also up-regulated RECK, a membrane glycoprotein that inhibits MMP-2 activity, and limited lung cancer cell invasion through coated filters [28]. Metacept-1 limited MMP-2 expression in treated leukemia cells [29]. Such anti-invasive activity could emerge through an epigenetic mechanism, but it could also arise via direct effects on proteins generally deemed necessary for invasion. Indeed, the structure of SAHA bound to an HDAC-like protein shows the hydroxamic acid group doubly coordinated to a zinc atom [30]. Several broad spectrum MMP inhibitors inhibit MMP activity in exactly the same manner, through binding MMPs via coordination to a zinc atom through a hydroxomate group [31].

While anti-invasive effects of HDAC inhibitors in a number of cell lines has been established, findings on these drugs' effects on cell motility (across uncoated substrates or through uncoated Transwell filters) are less consistent. TSA and SAHA were found to stimulate migration in Ishikawa cells [32]. TSA up-regulated integrins important in migration in hepatocellular carcinoma cells [33], and butyrate promoted migration in colon cancer cell lines [34]. On the other hand, TSA was found to have no effect on the motility of untransformed fibroblasts at concentrations at which invasion was strongly inhibited [26]. TSA has also been found to limit migration of hepatic stellate cells [35].

Given that motility and invasion are both essential in glioma's aggressive dispersion in brain tissue, we have investigated SAHA's effects on several rat and human glioma cell lines in 2D and 3D migration and invasion assays in vitro. The 2D assays employed allow independent interrogation of proliferation, migration, and invasion. The 3D assays allow interrogation of proliferation and invasion in an environment in which topology as well as cell–cell and cell–extracellular matrix contacts are similar to those in vivo. Such 3D studies are particularly important for study of HDAC inhibitors, as they regulate gene expression, and it is known that both gene expression and cell behavior can be starkly different for cells plated on 2D substrates vs. in 3D environments [36]. In particular, we focus on invasion assays in which glioma cells move through collagen I coated substrates or collagen I gels. We use collagen I as the structural protein in the invasion assays in part because collagen has been shown to be present at substantial levels in and around gliomas [37,38]. Additionally, one of the two primary modes of glioma invasion is along blood vessels, which are rich in collagen I and IV, two of the chief substrates of MMP-2 and MMP-9 [1,5]. Finally, gliomas invade very aggressively in collagen I gels in vitro, and such gels are increasingly being used as 3D environments in which to study glioma invasion [39–43].

In the 2D and 3D assays performed, we find that SAHA strongly inhibits cell proliferation but does not substantially induce cell death at concentrations up to 5.0 μM. Migration and invasion assays show that SAHA inhibits rat C6 and human U87 migration and invasion at $\leqslant$10.0 and 5.0 μM, respectively, when plated on 2D substrates. For multicellular tumor spheroids of 5 different glioma cell lines embedded in 3D collagen I matrices, SAHA strongly inhibits invasion at concentrations $\geqslant$2.5 μM. We also find that SAHA independently affects cell reorganization of the tumor surroundings and invasion into those remodeled surroundings. Preliminary investigation into the mechanisms by which SAHA limits glioma invasion shows that SAHA does not strongly bind secreted MMP-2 or MMP-9, despite the similarity in structure between SAHA and some MMP inhibitors. Moreover, zymography shows that MMP-9 is up-regulated in the presence of SAHA. This is consistent with quantitative real-time PCR analysis; however, such analysis also shows other proteases are down-regulated, and other genes important in cell–matrix interactions are affected. We suggest that increased cell–cell adhesion in the presence of SAHA may be the most important factor in SAHA's ability to limit invasion of glioma cells cultured in a 3D environment.

2. Materials and methods

2.1. Cells

WI-38 and 3T3 fibroblasts and LN18, F98, and F98EGFRvIII glioma cells were purchased from the American Type Culture Collection and cultured in medium per the supplier's instructions. C6 and U87 glioma cells were provided by Prof. Peter Canoll at Columbia University Medical School and cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B.

2.2. Drugs and chemicals

SAHA was provided by Prof. Ronald Breslow and diluted in DMSO. In all studies, an equivalent amount of DMSO without SAHA was added to the control culture medium. Dulbecco's modified eagle media (DMEM), Dulbecco's phosphate buffered saline (D-PBS), trypsin–ethylenediaminetetraacetic acid (trypsin–EDTA), FBS, calf serum, an antibiotic–antimycotic [containing penicillin, streptomycin, and amphotericin], and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen. A live/dead cell staining kit (L3224) used in 2D proliferation and viability assays and a CyQuant kit used in 3D proliferation and viability assays (7026) were also purchased from Invitrogen. Trypan blue solution (0.4%) was purchased from Sigma–Aldrich. Pepsin-solubilized type I collagen was purchased from Inamed Biomaterials. Collagenase type I (4000 U/mL) was purchased from Invitrogen (17100). Zymograph gels, buffers, Coomassie Blue R-250 Staining Solutions kit and SDS–PAGE standards were obtained from BioRad.

2.3. 2D proliferation and viability assay

A dye exclusion method was used to measure cell viability and proliferation. 5×10^4 cells were seeded in