Glioma invasion in vitro: regulation by matrix metalloprotease-2 and protein kinase C

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A hallmark of invasive tumors is their ability to effect degradation of the surrounding extracellular matrix (ECM) by the local production of proteolytic enzymes, such as the matrix metalloproteases (MMPs). In this paper, we demonstrate that the invasion of human gliomas is mediated by a 72 kDa MMP, referred to as MMP-2, and provide further evidence that the activity of MMP-2 is regulated by protein kinase C (PKC). The invasiveness of five human glioma cell lines (A172, U87, U118, U251, U563) was assessed in an in vitro invasion assay and was found to correlate with the level of MMP-2 activity (r² = 0.95); in contrast, the activity of this 72 kDa metalloprotease was barely detectable in non-invasive control glial cells (non-transformed human astrocytes and oligodendrocytes). Treatment with 1,10-phenanthroline, a metalloprotease inhibitor, or with a synthetic dipeptide, containing a blocking sequence (ala-phe) specific for MMPs, resulted in a > 90% reduction in glioma invasion. Furthermore, this MMP-2 activity could be inhibited by the treatment of tumor cells with calphostin C, a specific inhibitor of PKC. Glioma cell lines treated with calphostin C demonstrated a dose-dependent decrease (IC₅₀ = 30 nM) in tumor invasiveness with a concomitant reduction in the activity of the MMP-2. Conversely, treatment of non-invasive control astrocytes with a PKC activator (phorbol ester) led to a corresponding increase in their invasiveness and metalloprotease activity. These findings support the postulate that MMP-2 activity constitutes an important effector of human glioma invasion and that the regulation of this proteolytic activity can be modulated by PKC.

Keywords: glioma, invasion, metalloprotease, protein kinase C, zymography

Introduction

Despite many therapeutic strategies undertaken in the treatment of glioblastoma multiforme (GBM), the survival rate for patients afflicted with this aggressive cerebral malignancy remains poor [1]. Even with combinations of different therapeutic modalities, a good prognosis is extremely rare, as remaining cells infiltrating the normal brain tissue inevitably lead to tumor recurrence [2]. The development of such local invasion remains an important cause of morbidity and mortality, thus underscoring the need to further understand and target the cellular mechanisms that underlie tumor invasiveness.

A common feature of the invasive process is the degradation of the extracellular matrix (ECM) by an array of proteolytic enzymes expressed by the infiltrating tumor cells [3,4]. Matrix metalloproteases (MMPs), capable of degrading almost all ECM components, have been suggested to play an important role in mediating tumor invasion [5–7]. In this regard, the elevated expression of various MMPs by many peripheral tumors is strongly associated with the invasive phenotype [3,5,7–13]. MMP expression has also been demonstrated in...
invasive cerebral tumors [14–18]. A MMP secreted by the rat glioma cell line BT5C was determined to be capable of degrading fetal rat brain aggregates [14,19]. The invasion of another rat glioma, C6, into the optic nerve has been shown to be inhibited by 1,10-phenanthroline, a MMP inhibitor [18]. In vitro, human glioma cell lines have been shown to express a variety of MMPs (collagenases corresponding to 54, 65, 72 and 92 kDa) [20,21]. Similarly, in vivo, immunohistochemical localization studies have confirmed that high-grade human gliomas (GBM and anaplastic astrocytoma) synthesize these collagenases, whereas non-invasive low-grade astrocytomas and normal brain tissue do not [16]. It is unclear which of these MMPs is primarily responsible for mediating glioma invasion.

The present study was undertaken to determine whether MMPs expressed by gliomas regulate the invasive phenotype. In a survey of five human glioma cell lines that differ with respect to their invasiveness in an in vitro assay system, we demonstrate the existence of a strong correlation between glioma invasion and the expression of the 72 kDa collagenase, also referred to as MMP-2 or gelatinase A. Furthermore, as we have previously reported that gliomas express abnormally high levels of PKC activity compared to their non-transformed glial counterparts [22–25], we investigated how modulation of this signal transduction pathway affects glioma invasion. We report that pharmacologic inhibition of PKC in gliomas reduces both MMP-2 activity and invasion. Conversely, treatment of non-invasive human astrocytes with a phorbol ester to stimulate PKC resulted in an increased MMP-2 activity and invasion. The data support the hypothesis that the invasive phenotype of high-grade gliomas is modulated by MMP-2, which in turn is regulated by PKC.

Materials and methods

Cell culture and treatments
All human glioma cell lines (A172, U87, U118, U251, and U563) were obtained from American Type Culture Collection [26]. All lines were regularly passaged by treatment with trypsin (0.05%) and were grown in feeding medium of Eagle’s minimum essential medium supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin, non-essential amino acids, 1 mg/ml glucose, and 1 mM pyruvate. We routinely confirm that all our cell lines are free of mycoplasma contamination using the Stratagene Mycoplasma PCR Kit.

Human fetal astrocytes were utilized as non-malignant control populations. These cells were isolated, characterized, and maintained in culture as previously published by our laboratory [27]. In brief, neural tissue from therapeutic abortions (10–18 weeks) was obtained in accordance with local Ethics Review Committee. Following the removal of the meninges, the neural tissue is crudely dissociated by trituration and then incubated in 0.25% trypsin and 100 μg/ml DNAse for 30 min at 37°C. Cells are further dissociated by filtration through a 130 μM mesh and then pelleted by centrifugation. These cells are then resuspended in feeding medium and plated onto poly-L-lysine coated 100 mm dishes. After 1 week, cells are removed with trypsin and replated twice weekly thereafter. One month post-initial plating the cells are considered ready for use and the purity of fetal astrocytes was assessed by immunoreactivity to glial fibrillary acidic protein (GFAP) and determined to exceed 95%. The minor contaminating cell population consists of fibroblasts, believed to originate from the meninges. These fetal astrocytes are adherent, exhibit a rapid growth rate when compared with adult astrocytes, and are contact inhibited.

Cell treatments were performed following standard methodologies with few exceptions. Although treatments were normally applied to cells 2 h post-seeding, in the case of peptide applications, both peptides (1 mM final concentration) were solubilized in dimethylsulfoxide (DMSO) and applied to cells in suspension for a period of 15 min prior to seeding onto invasion chambers. The final concentration of DMSO in the invasion assay did not exceed 0.3% and untreated controls were incubated in an equivalent concentration of vehicle. Furthermore, as previous reports have demonstrated that the inhibitory effect of calphostin C on PKC requires photoactivation [28], all cultures treated with this agent were exposed to a fluorescent light source for a 45-min period. Since calphostin C was initially solubilized in absolute ethanol, all treatment groups (including controls) received equal amounts of vehicle (0.1% ethanol).

Invasion assay
The in vitro invasion assay was modified from the methods described by Albini et al [29]. Briefly, Biocoat Matrigel (Collaborative Biomedical, Bedford, MA, USA) consists of two compartments separated by a filter (9 mm in diameter, with 8 μg pore size) precoated with Matrigel (100 μg/cm²), a reconstituted extracellular matrix preparation, whose constituents consist of collagen type IV, laminin,