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Original Paper

The Effect of Exogenous Gangliosides on Matrix Metalloproteinase Secretion by Human Glioma Cells *In Vitro*

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Matrix metalloproteinases (MMPs) are zinc-dependent peptidases and are amongst those enzymes responsible for extracellular matrix (ECM) degradation during tumour-cell migration. Gangliosides are a family of acidic membrane glycolipids thought to play a role during cell development, differentiation and oncogenic transformation. In this descriptive study, we investigated the effects of six exogenous gangliosides (GM1, GM3, GD1a, GD1b, GD3 and GT1b) on the secretion of MMP-2 (72 kDa gelatinase or gelatinase-A) and MMP-9 (92 kDa gelatinase or gelatinase-B). Cell-conditioned media from eight human glioma-derived cell-lines served as the source of MMPs and were investigated using SDS–PAGE zymography. Six of the cell lines showed upregulation of secretion of both enzymes by all six gangliosides. Of the remaining two cell lines, one showed inhibition of MMP secretion by all gangliosides and the other had a small but differential reponse to the range of gangliosides investigated. These results suggest that gangliosides may stimulate glioma cell invasiveness by promoting MMP expression. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

INTRINSIC TUMOURS of the central nervous system, of which gliomas constitute more than 50%, are characterised by their ability to infiltrate the adjacent brain parenchyma in a diffuse manner, often several millimetres beyond any obviously defined tumour margin. This property accounts for the high incidence of recurrence even following drastic surgical resection. Furthermore, such tumours exhibit a high degree of cellular heterogeneity and are consequently highly adaptive against existing therapeutic strategies, resulting in poor overall prognosis for the patients.

The mechanisms underlying brain tumour cell invasion are not well understood. However, a three-step hypothesis for invasion, in general, was proposed by Liotta and colleagues [1, 2], suggesting that there is initial attachment of the invading cell to elements of the extracellular matrix (ECM), proteolytic degradation of the ECM and subsequent movement of the cell into the resulting area of compromised matrix integrity. Less well documented are the mechanisms which regulate these processes.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases which have broad specificities for ECM proteins. MMPs are synthesised as proenzymes and generally secreted upon demand, probably upon cell stimulation with growth factors, cytokines or oncogene expression. They are active during embryogenesis and tissue remodelling, in pathological connective tissue conditions, such as arthritis and osteoporosis and are generally believed to facilitate cancer cell invasion and metastasis [3]. The MMP family includes at least 11 distinct members and these are divided into three broad subclasses according to their substrate specificities: the collagenases, gelatinases (type IV collagenases) and stromelysins [3, 4]. A major mechanism for MMP regulation *in vivo* is via their binding to specific tissue inhibitors of metalloproteinases (TIMPs). Three have been identified (TIMPs 1, 2 and 3), they have a broad range of specificity for MMP enzymes, with which they interact *in vitro* in a 1:1 stochiometric ratio [5]. TIMP-1 is a 28 kDa glycoprotein which can form a complex with the active form of MMP-9; TIMP-2 is unglycosylated and binds to both latent and active forms of MMP-2

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[6]. TIMP-3, the most recently described, is also unglycosylated and is related to, but distinct from, ChIMP-3 found in chicken embryos [7].

Gangliosides are acidic glycosphingolipids characterised by the presence of one or more sialic acid (*N*-acetyl neuraminic acid) residues in their oligosaccharide chain; they are found in the membranes of all eukaryotic cells, but are particularly abundant in the central nervous system [8], especially during development, when an increase in their total complement has been shown to correlate with axonal and dendritic outgrowth and synaptogenesis. Their proposed functions include cell–cell/cell–substrate recognition and binding [9], modulation of cell membrane receptor function, including cytokine and growth factor receptors [10, 11] and the control of cell proliferation [12]. In addition, most gangliosides inhibit the action of mitogens *in vitro*.

It has been observed that, whereas the total ganglioside complement is higher in normal brain than in neoplastic brain tissue, the proportion of structurally simple gangliosides is increased in the latter with respect to the more complex, normal brain gangliosides [13]. The proportion of the simple ganglioside GD3 has been shown to correlate directly with histologically defined malignancy [14, 15] and in malignant melanoma an anti-GD3 monoclonal antibody has been shown to elicit tumour regression [16]. GD3 and GD2 have also been implicated in cell binding to components of the extracellular matrix both in human melanoma cells [17] and human glioma cells [18]; cell-ECM binding being an important initial step in tumour cell invasion.

During *in vitro* assays gangliosides have been shown to promote the invasive behaviour of neoplastic glia [12]. Moreover, invasive cells express gangliosides recognised by the monoclonal antibody A2B5 [19] during their migratory phase and it seems likely, therefore, that gangliosides may play a pivotal role in the regulation of cell invasion mechanisms. Since the expression of simple gangliosides is known to be lost rapidly with sequential *in vitro* passage, we investigated the effect of six exogenously applied gangliosides (GM1, GM3, GD1a, GD1b, GD3 and GT1b) on the secretion of two MMPs in eight glioma derived cell lines. The MMPs investigated were MMP-2 (72 kDa gelatinase or gelatinase A) and MMP-9 (92kDA gelatinase or gelatinase B).

MATERIALS AND METHODS

Cell culture

The cell lines used in this study are described in Table 1. Cells were cultured as monolayers in six-well plastic culture plates (Marathon) at 37°C, 5%CO_2 in a standard humidified incubator. Cells were initially maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco BRL) and 1% antibiotic/antimycotic. This medium was discarded as the monolayers attained 70% confluence and replaced on sequential days with media supplemented with 7%, 3% and finally serum-free media containing one of the following gangliosides at 100 ng/ml: GM1, GM3, GD1a, GD1b, GD3 and GT1b. All gangliosides were obtained from Sigma as lyophilised powders and reconstituted in DMEM to give stock solutions of 100 μg/ml. The cells were incubated with gangliosides under serum-free conditions for 48 h whereupon the media were harvested and freeze-dried. These cell-conditioned medium samples served as the source of metalloproteinases for the experiments. They were freeze-dried and reconstituted in an appropriate volume of sterile water according to the number of cells in the monolayer from which they had been harvested to provide a standard of 10^6 cells/ml of reconstituted medium.

Table 1. Cell lines investigated

Cell-line designation	Passage number	Histological diagnosis
IPTR-6	8	Disembryoplastic neuroepithelial tumour (DNET)
IPPS-11	6	Ependyoma
IPNT-H	10	Grade I/II pilocytic astrocytoma
IPSB-18	31	Anaplastic astrocytoma
GO-G-CCM	>100	Anaplastic astrocytoma
GO-G-UVW	>100	Anaplastic astrocytoma
IPRM-5	9	Glioblastoma multiforme
IPJM-6	5	Glioblastoma multiforme

All cell lines with the 'IP' prefix were derived from material taken at biopsy in the neurosurgical unit at the Maudsley Hospital, London. GO-G-CCM and GO-G-UVW were a generous gift from Ian Freshney at the CRC laboratories, University of Glasgow, Scotland.

Electrophoresis

Zymogram analysis was performed by a modification of the method of Heussen and Dowdle [20] to investigate the activities of the two gelatinases. Briefly, a gelatin substrate was co-polymerised into an 11% acrylamide resolving gel at the time of gel casting. Aliquots (60 μl) of cell-conditioned media were incubated for 1 h at 37°C with 5 μl of 20 mM *p*-aminophenyl mercuric acetate (APMA), an organomercurial activator of MMP proenzymes. The aliquots were then mixed with 15 μl of a loading buffer (Tris base) containing glycerol (30%), sodium dodecyl sulphate (SDS at 7.7%) and bromophenol blue (0.03%) at pH 6.8 before being loaded into stacking gel wells. Following electrophoresis in a Mini Protean II Dual Slab Cell (Bio Rad), the gels were immersed in Triton X-100 (2.5%) for 1 h to remove the SDS. The gels were incubated in a 50 mM Trizma–HCl buffer (pH 7.6) containing 10 mM $CaCl_2$ at 37°C for 24 h before being stained overnight with 0.075% Coomassie brilliant blue in methanol/acetic acid. Finally, the gels were destained with methanol/acetic acid until bands became visible; proteolytic activity was indicated by the appearance of light bands against the dark blue background (see Figure 1). The enzyme activity observed was confirmed to be attributable to MMPs by repetition of the experiments using the incubating buffer containing 10 mM, 1,10-phenanthroline, a specific inhibitor of MMP enzymes.

RESULTS

A representative gel of a zymogram assay is shown in Figure 1.

Six of the eight cell lines used (IPTR-6, IPPS-11, IPRM-5, IPJM-6, GO-G-CCM and GO-G-UVW) exhibit upregulation of both metalloproteinases by all six of the gangliosides investigated. Conversely, the secretion of both gelatinases in the IPSB-18 cell line (derived from an anaplastic astrocytoma) was downregulated by all of the gangliosides tested. The IPNT-H cell line (derived from a low grade pilocytic astrocytoma) showed no secretion of MMP-2 (72 kDa

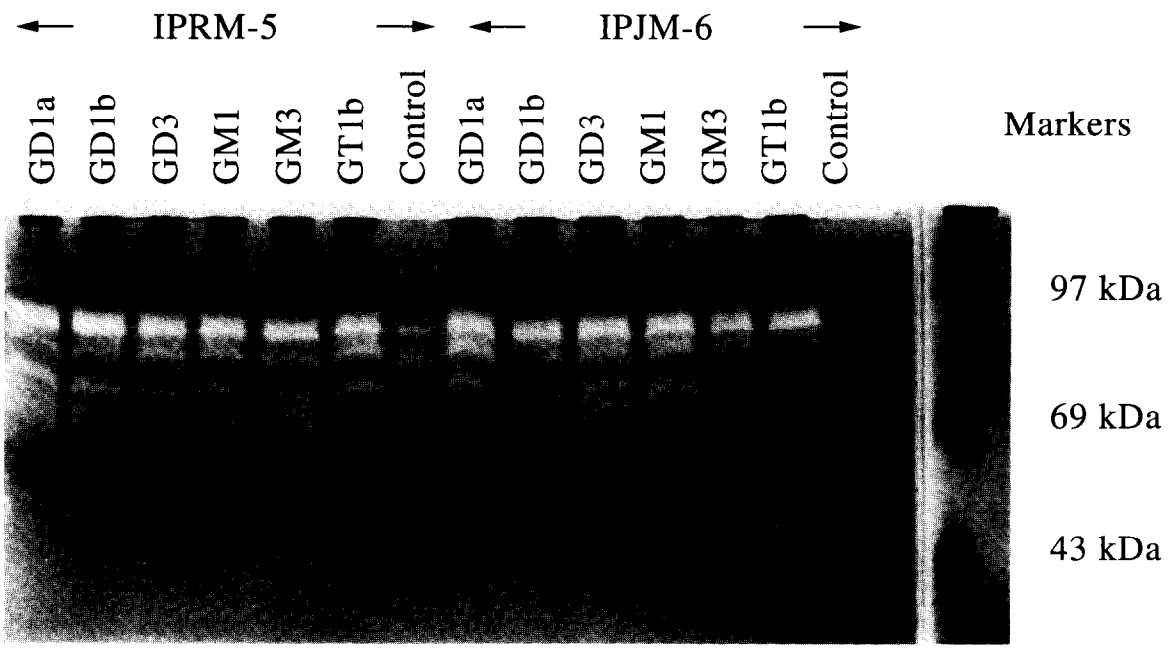

Figure 1. A representative gel of a zymogram assay for the two glioblastoma multiform-derived cell lines IPRM-5 and IPJM-6. The 92 kDa gelatinase (MMP-9) appears as a well-defined set of doublet bands above the less intense 72 kDa gelatinase (MMP-2) bands. The doublet band configuration reflects the presence of both the activated and latent forms of the enzymes; both enzymes can be seen to be upregulated by gangliosides with respect to the controls. Molecular weight markers (Amersham) shown on the right of the figure were phosphorylase-b (97 kDa), bovine serum albumin (69 kDa) and ovalbumin (43 kDa).

gelatinase), either in controls or with ganglioside treatment. It does secrete small amounts of MMP-9 (92 kDa gelatinase), but this was unaffected by GD1a, slightly up-regulated by GD1b, GD3, GM1 and GM3 and totally inhibited by GT1b. All experiments, when repeated in the presence of the MMP inhibitor 1,10-phenanthroline showed total ablation of enzyme activity, thereby confirming the activity to be attributable to MMPs.

We emphasise that the techniques used in the study are qualitative and no attempt has been made to quantify the relative effects of different gangliosides.

DISCUSSION

The results indicate that the gangliosides investigated are capable of upregulating the secretion of matrix metalloproteinases *in vitro*. These findings substantiate previous studies by our group [12] which have demonstrated that the same gangliosides (with the exception of GM1) differentially downregulate cell proliferation and promote cell migration and invasion. It would seem that gangliosides modulate the invasive potential of these cell lines by both upregulating ECM degradation and synthesis and probably also by acting as cell-matrix adhesion molecules [18, 21].

The precise molecular mechanisms responsible for these effects are yet to be elucidated but may relate to the ability of gangliosides to modulate the activity of membrane receptor proteins. Previous studies have indicated that the simple ganglioside GM3 and its derivatives can inhibit EGF-stimulated phosphorylation of the EGF receptor and thereby inhibit EGF-stimulated cell proliferation [22, 23], while the effect of GM3 has also been reported to be biphasic:inhibitory at low concentrations and stimulatory at high concentrations [24]. However, the GM3 derivative, de-*N*-acetyl GM3, strongly enhances the kinase activity associated with the EGF receptor [25]. Thus GM3 de-acetylation mechanisms may have a close regulatory effect on the EGF receptor, the gene for which is amplified in over 30% of gliomas [26]. Similarly, dimerisation and autophosphorylation of the PDGF receptor has been shown to be inhibited by GM1, GM2, GM3, GD1a and GT1b [10, 27].

Growth factors are known to alter membrane gangliosides in cultured neoplastic glia [17, 28, 29] and they have also been demonstrated to regulate metalloproteinase secretion [30, 31]. The principal control mechanism of MMP production is probably growth factor-mediated since, in general, MMPs are not consitutively expressed but produced upon demand. The mechanism of EGF and PDGF promotion of MMP secretion involves induction of the *C-FOS* and *C-JUN* genes, the protein products of which form a heterodimer generally referred to as AP-1 transcription factor. AP-1 can bind to the promoter sequence of the MMP-9 gene at a site known as the TPA responsive element and induce transcription [5, 32]. The promoter of MMP-2 does not possess a TPA responsive element, but does have a binding site for the AP-2 transcription factor; the latter mediating signal transduction in the protein kinase C pathway which has been shown to be modulated by simple gangliosides [33].

The differential effects of gangliosides on MMP secretion in our cell lines may be due to differential expression of growth factor receptors in these cells, although the mechanisms for MMP induction in ganglioside-treated cells may be different from the growth factor stimulated pathway in proliferating cells. One possibility is that gangliosides downregulate expression of the tissue inhibitors of metalloproteinases (TIMPs) and this is currently being investigated in our laboratory. It is interesting to note that, in general, the histological type of the tumours from which the cultures were derived did not appear to be correlated with either MMP secretion or

response to ganglioside treatment. The IPNT-H line was derived from a large pilocytic astrocytoma of the hypothalamus in a 6 month old child which, despite its low histological grade of malignancy, displayed a high mitotic index and grew predominantly by expansion, with little evidence of diffuse invasion *in vivo*. The low levels of MMP and the poor response to gangliosides may, therefore, reflect this lack of invasive phenotype. The other line which differed from the norm, IPSB-18, is unusual in that its component cells are well differentiated, even at high passage and continue to express high levels of glial fibrillary acidic protein GFAP [34]. Such a uniquely well differentiated glioma line may be expected to display a different MMP profile.

The results presented in this report augment those already obtained in our laboratory [12] and further illustrate the significance of gangliosides in brain tumour biology.

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