Inhibitory effects of retinoic acid and IIF on growth, migration and invasiveness in the U87MG human glioblastoma cell line

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Received April 19, 2007; Accepted June 7, 2007

Abstract. Glioblastomas, the most malignant and prevalent brain tumors which remain incurable, are characterized by both extensive proliferation and invasive growth. We previously reported a remarkable antitumoral effect of the retinoid 6-OH-11-O-hydroxyphenanthrene (IIF) on neuroblastoma, leukemia and colon carcinoma cells. In this study we examined the effect of IIF on proliferation, apoptosis and cell invasion in the human glioblastoma cell line U87MG, in comparison with all-trans-retinoic acid (RA). Our results showed that both retinoids induced cell growth inhibition and apoptosis in a dose- and time-dependent manner. We also demonstrated that the invasive ability of glioblastoma cells decreased after treatment with IIF or RA. Since cell invasion involves a complex system of tightly regulated proteases, matrix metalloproteinases (MMPs) and their specific inhibitors, tissue inhibitors of MMPs (TIMPs), we analysed the effect of IIF on MMP and TIMP expression in comparison with RA. Treatment with both retinoids resulted in a marked decrease of MMP2 and MMP9 expression and of lytic activity of MMP2. In addition, exposure to IIF led to enhanced expression of TIMP2. Collectively, our results demonstrated the effectiveness of both IIF and RA in inhibiting proliferation, cell migration, and the invasive potential of glioblastoma U87MG cells. Notably, the anticancer activity of IIF, on the whole, was more pronounced than that of RA. Therefore, these findings, besides providing further evidence that IIF may be a powerful tool in the development of cancer treatments, suggest that IIF may have therapeutic potential against the invasiveness of brain tumors.

Introduction

Gliomas are the most prevalent primary brain tumors, accounting for >50% of all brain tumors (1-2). Current treatment options include surgery, radiation therapy, and chemotherapy. Unfortunately, the prognosis of patients with glioblastomas remains extremely poor, and the median survival of 12 months from the time of diagnosis has not significantly changed during the last few years (3-4). New approaches are therefore essential for the treatment of these patients, especially because the occurrence of gliomas is increasing. Invasive behaviour is the pathological hallmark of malignant gliomas, being responsible for the failure of surgery, radiation and chemotherapy (5-6).

Cell invasion is a multistep cellular process in which a major role is played by the proteolysis of the extracellular matrix (ECM) components, such as collagen type IV and laminin by matrix metalloproteinases (MMPs). In particular, the expression and proteolytic activity of the so-called gelatinases, MMP2 and MMP9, are also well correlated with the progression and the degree of malignancy in gliomas (7-11).

The activity of MMPs is finely regulated by tissue metalloproteinase inhibitors (TIMPs); an imbalance between the production of MMPs and their inhibition by TIMPs, leading to excess degradation of the ECM, underlies the invasive potential of brain tumors (12-14). In particular, TIMP1 and TIMP2 regulate MMP9 and MMP2 respectively. Thus, inhibitors of MMPs could potentially be useful for blocking cancer progression.

Retinoids, a group of natural and synthetic analogs of vitamin A, play an important role in cell proliferation and differentiation (15). Because retinoid treatment inhibits the growth of a variety of epithelial cancers, retinoids have great promise in the area of cancer chemotherapy and chemoprevention (16-18). However, the use of these agents is significantly hampered by their toxicity and the emergence of resistance (19,20). Hence major efforts are underway to detect new retinoids which could exhibit similar anticancer activities but with reduced toxicity in vivo. The retinoid signal is mediated through specific nuclear receptors: the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). It has been demonstrated that retinoids which are specific ligands of RXR are effective as antitumoral agents as RAR

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Key words: glioblastoma, IIF, invasion, retinoids, metalloproteinases
ligands, and are devoid of side effects (21). Therefore, many retinoids able to bind RXR with high affinity have been synthesized, among which is the derivative of all-trans-retinoic acid (RA), 6-OH-11-O-hydroxyphenantrene (IIF) (22).

In previous studies we demonstrated that this compound had a strong antitumoral effect on neuroblastoma, leukemia and colon carcinoma cells (22-25).

In this study we investigated the anticaner properties of IIF in the human glioblastoma cell line U87MG, in comparison with the primary biologically active retinoid RA by analyzing the effect of both retinoids on cell proliferation, on the induction of apoptosis and on the expression of genes and proteins involved in the mechanism leading to tumor invasion.

**Materials and methods**

**Cell culture.** The human tumor cell line U87MG, obtained from Interlab Cell Line Collections (Genoa, Italy), was maintained in RPMI (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin and grown at 37°C in humidified air with 5% CO2.

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**Western blot analysis.** To determine MMP2, MMP9, TIMP1, TIMP2 and Bcl2 levels, U87MG cells were plated and treated with retinoids as described above for zimography. The cells were detached and were collected by centrifugation at 300 x g for 10 min, and the pellets were re-suspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5% Triton X-100, and 5 μM Na2VO4) and sonicated on ice in the presence of protease inhibitors. The protein concentration was determined by the method of Lowry (29). Cell lysates (50 μg of protein per lane) were size-fractionated in 10% SDS-polyacrylamide gel prior to transfer to Hybond™-C Extra membranes (GE Healthcare, Milan, Italy) according to standard protocol. Membranes were blocked for 2 h with 5% milk in transfer buffer saline (TBS) at RT. The membranes were incubated overnight at 4°C with anti-MMP2, anti-MMP9, anti-TIMP1, anti-TIMP2, or anti-Bcl2 dissolved in TBS-5% milk. All the antibodies employed were from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The membranes were washed two times with TBS-5% milk and were incubated for 1 h with the respective peroxidase-conjugated antibodies. The anti-MMP2 and the anti-MMP9 were diluted 1:700; the anti-TIMP1 and the anti-TIMP2, 1:400; the anti-Bcl2, 1:300; and the anti-rabbit peroxidase-conjugated antibodies were diluted 1:1000, respectively with 5% milk in TBS-1% Tween. The proteins were detected by luminol (GE Healthcare). Bands were quantified by using a densitometric image analysis software (Image Master VDS, Pharmacia Biotech, Uppsala, Sweden). No MMP9 activity was exhibited in the gel under these experimental conditions.

**MMP activity.** The MMP2 activity of the U87MG cells was determined by gelatin zimography (28). After a 4-day treatment with RA (10-20 μM) or IIF (10-20 μM), the cells were washed with PBS and replaced in serum-free medium (RPMI) for 18 h with the same treatment. After centrifugation (300 x g for 10 min) the supernatant was separated and the protein concentration was determined; 10 μg per lane added to sample buffer (1 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate (SDS), and 10% glycerol] was applied to 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin (Sigma). After electrophoresis, SDS was removed from the gel by washing twice with 2.5% Triton X-100 for 1 h. After a brief rinse, the gel was incubated at 37°C for 18 h in buffer, pH 7.6, containing 100 mM Tris-HCl, 10 mM CaCl2, and 20 mM NaCl. The gel was stained with 1% Coomassie Brilliant Blue R250 for 2 h and then destained with destaining solution (40% methanol, 10% acetic acid, and 50% distilled water). The MMP2 activity was discernible by clear bands of gelatin digestion on a blue background. The activity of MMP2 in the gel was quantified by using a densitometric image analysis software (Image Master VDS, Pharmacia Biotech, Uppsala, Sweden). No MMP9 activity was exhibited in the gel under these experimental conditions.

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GAGTTGTGG-3' (Tm, 55.5˚C); MMP9 reverse, 5'-GGTGA AGGGGAAGTAGAAGC-3' (Tm, 55˚C); GUSB forward, 5'-TGGTATAAGAAGTATCAGAAGCC-3' (Tm, 53˚C); and GUSB reverse, 5'-GTATCTCTCTCGCAAAAGGAAC-3' (Tm, 54.4˚C).

MMP2 and MMP9 mRNA levels for each sample were normalised against ß-glucuronidase (GUSB) mRNA levels, and relative expression was calculated by using Ct values. Real-time PCR was performed by using SYBR-Green Supermix and the IQ Cycler thermocycler (Bio-Rad).

Invasion assay. The invasion of cells into Matrigel was determined using Boyden chambers (NTG, Milan, Italy) with an 8.0-μm pore size polycarbonate membrane (NTG, Milan, Italy) (30). The filter was coated with 12.5 μg of Matrigel (Sigma). After 2 h, U87MG cells, treated or not with 20 μM RA or 20 μM IIF for 4 days, were seeded into the upper part of each chamber (4x10^5 cells/well in 800 μl serum-free RPMI) with the same treatment. In the lower part of the chamber, 200 μl chemoattractant (RPMI with 10% FBS) was added. After incubation for 18 h at 37˚C, non-migratory cells on the upper surface of the filter were wiped with a cotton swab, and the cells which migrated to the lower surface of the filter were fixed and stained with Toluidine blue. The invasive activity was evaluated by counting cells in five random fields using microscopy at x100 magnification.

Statistical analysis. Data were expressed as the mean ± SE. Differences were analysed by the Student’s t-test and considered statistically significant at p<0.05 between the control and experimental samples.

Results

The effects of IIF and RA on U87MG cell growth, measured by MTT assay, are shown in Fig. 1. Both retinoids reduced cell proliferation, in a dose- and time-dependent manner. IIF exhibited a higher growth inhibitory effect than RA at equimolar doses.

Growth inhibition was accompanied by changes indicating that cells underwent apoptosis. Apoptotic cells were detected by the Annexin V test. FITC-labeled Annexin V binds the newly exposed phosphatidylserine (PS) at the outer membrane leaflet. In untreated samples, the amount of apoptotic cells was 3%. Treatment with 40 μM IIF or RA increased this percentage respectively to 56 and 40% (Fig. 2).

Bcl2 overexpression has been demonstrated in many cancer cells where its downregulation is indicative of apoptosis. We therefore evaluated the effect of IIF and RA on Bcl2 protein expression (Fig. 3). As demonstrated by Western blot analysis, both retinoids caused a dose-dependent downregulation of the Bcl2 protein, IIF being more effective than RA.

Invasion Matrigel assay was performed in order to determine the ability of retinoids to inhibit tissue invasion (Fig. 4). Our results showed that treatment with retinoids was followed by a decrease in the invasive activity of U87MG cells; 20 μM IIF caused a 49% reduction in the migrating cells with respect to the controls, while a 41% reduction was obtained using RA at the same dose. A dose of 20 μM was chosen in order to avoid possible misinterpretation of the results due to massive cell death occurring when higher doses of retinoids are used.
Since MMPs play a major role in tumor cell invasion, and MMP2 and MMP9 were shown to be upregulated in gliomas, we evaluated the effect of IIF and RA on their expression. As demonstrated by Western blot assay (Fig. 5), MMP2 and MMP9 expression levels were reduced by 30% with respect to the control when 30 μM IIF was employed and 20% after treatment with RA at the same dose.

This result was confirmed by real-time PCR: both retinoids induced inhibition of MMP mRNA levels. As shown in Fig. 6, 20 μM IIF induced a 42% decrease in MMP2 mRNA and a 20% decrease in MMP9 mRNA levels, while RA induced a 30% decrease in MMP2 and a 25% decrease in MMP9 mRNA levels.
39% decrease in MMP9 mRNA. After treatment with RA at the same dose, MMP2 and MMP9 mRNA was reduced by 20% (not shown).

We also examined the effect of IIF and RA on MMP2 enzymatic activity. As measured by gelatin zymography, a decrease in gelatinolytic activity of MMP2 was observed after exposure to IIF or RA, IIF being more effective than RA (Fig. 7).

Finally, in order to determine whether the exerted effect of IIF and RA on MMPs was accompanied by an effect on TIMPs, we analysed TIMP1 and TIMP2 expression levels by Western blotting. Notably, as shown in Fig. 8, treatment with 30 µM IIF resulted in a 40% increase of TIMP2 and a 15% increase of TIMP1 protein level, while treatment with the same dose of RA significantly increased only TIMP1 expression.

Discussion

Retinoids have long been investigated in preclinical models, and clinical data have already supported the potential of these...
compounds as cancer preventive and therapeutic agents (15-18). In previous studies we demonstrated the strong antitumor activity of the retinoid IIF on many cancer cell lines, and its activity was consistently stronger than that exerted by RA (22-25).

The objective of the present research was to assess the potential of IIF as an anticancer agent on U87MG glioblastoma cells in comparison with RA.

The above reported data demonstrated that IIF had a strong antiproliferative effect, always higher than that exerted by RA. This effect was accompanied by a proapoptotic action. During apoptosis, the loss of phospholipid asymmetry occurs, which results in exposure of phosphatidylserine (PS) residues at the outer plasma membrane, which may serve as a trigger for phagocytosis (31). The exposure of PS has been demonstrated to occur early during the effector phase of apoptosis, probably downstream of changes in the mitochondria, and precedes the loss of plasma membrane integrity and DNA fragmentation (32). Treatment with RA and IIF induced a PS exposure, still detectable after 24 h as demonstrated by the Annexin V test.

Overexpression of the antiapoptotic protein BcI2, reported in a wide variety of cancer cells, is associated with a diminished apoptotic response and resistance to various antitumor agents (33,34). BcI2 blocks apoptosis by inhibiting the release of mitochondrial proteins and PS exposure (32). Our results indicated that IIF and RA caused a downregulation of BcI2 protein in U87MG cells.

This observation is particularly important because it was demonstrated that BcI2 also promotes migration and invasiveness in human glioma cells (35). Invasive behaviour is the pathological hallmark of malignant gliomas and consequently the invasive cascade is an important therapeutic target. Invasion of tumor cells into normal tissue involves the interaction of tumor cells with ECM and surrounding cells, and their ability to secrete matrix-degrading proteases. Several models have been used to study invasive behaviour, the most widely used being the Matrigel invasion assay. By using this assay, we found a dose-dependent decrease in glioblastoma cell invasion by both retinoids, confirming the ability of retinoids to inhibit the invasive capacity (36,37).

MMPs are thought to play a critical role in the invasive behaviour of glioblastoma. Particularly, MMP2 and MMP9 were found upregulated and also were associated with the degree of malignancy of gliomas (38-40), suggesting that these enzymes could be a molecular target in the treatment of glioma. In light of this, we investigated the effect of IIF, in comparison with RA, on MMP2 and MMP9 by Western blot, real-time PCR and gelatin zimography analyses. We found that retinoids inhibited MMP2 and MMP9 expression at both the mRNA and protein level, suggesting that the above reported inhibitory effect of retinoids on cell invasion could be due, at least in part, to the downregulation of MMP2 and MMP9 expression.

Furthermore, both retinoids dramatically reduced the enzymatic activity of MMP2. In our experimental conditions we were able to detect only negligible amounts of MMP9 gelatinolytic activity. Comparatively, in gliomas, MMP2 activity is very high with respect to that of MMP9 (41).

The catalytic activity of MMPs is closely regulated by their specific inhibitors, TIMPs. Changes in TIMP levels directly affect MMP activity since they have the ability to form tight 1:1 complexes with the active MMP enzymes (42-45). Therefore, in the present study, we investigated whether IIF and RA also affected the expression of TIMP1 and TIMP2, endogenous regulators of MMP9 and MMP2 respectively. Notably, we found that exposure to both retinoids, especially IIF, resulted in upregulation of TIMP2 expression.

The ability of glioma cells to infiltrate the surrounding brain tissue and escape current therapeutic modalities could potentially be minimized using anti-invasive therapies (46). Collectively, our results demonstrate the effectiveness of the retinoids IIF and RA as anticancer agents in glioblastoma U87MG cells. Remarkably, IIF exhibited heightened effectiveness over RA, in that it exerted major effects on growth, apoptosis, migration and invasive potential. It has been demonstrated that retinoids which are specific ligands for RAR are effective as antitumoral agents as RAR ligands, and are devoid of side effects (21). Our findings suggest that IIF, a ligand of RXR (22), may have good therapeutic potential in the treatment of brain tumors.

Acknowledgements

This study was supported by a grant from MURST, Italy. We thank Dr A. Strillacci for his support with the real-time PCR assay and Dr A. Santon for his support with the Annexin-V assay.

References


