The Relationship between Intracellular Protein Kinase C Concentration and Invasiveness in U-87 Malignant Glioma Cells

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Abstract

Objective: Glioblastomas, the most common type of primary brain tumors, are highly invasive and cause massive tissue destruction at both the tumor invading edges and in areas that are not in direct contact with glioma cells. As a result, patients with high-grade gliomas are faced with a poor prognosis. Such grim statistics emphasize the need to better understand the mechanisms that underlie glioma invasion, as these may lead to the identification of novel targets in the therapy of high grade gliomas. Protein kinase C (PKC) is a family of serine/threonine kinases and an important signal transduction enzyme that conveys signals generated by ligand-receptor interaction at the cell surface to the nucleus. PKC appears to be critical in regulating many aspects of glioma biology. The purpose of this study was to assess accurately the role of PKC in the invasion regulation of human gliomas based on hypothesis that protein kinase C (PKC) is functional in the process of glial tumor cell invasion.

Method: To test this hypothesis, U-87 malignant glioma cell line intracellular PKC levels were up and down regulated and their invasiveness was tested. Intracellular PKC level was characterized using PKC activity assays. Invasion assays including barrier migration and spheroid confrontation were used to study the relationship between PKC concentration and invasiveness.

Result: The cell line which were treated by PKC inhibitor tamoxifen and hypericin exhibited decreased PKC activity and decreased invasive abilities dose dependently both in matrigel invasion assay and tumor spheroid fetal rat brain aggregates (FRBA) confrontation assay. However, the cell line that was treated by PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA) did not exhibit increases in either PKC activity or invasive ability.

Conclusion: These studies suggest that PKC may be a useful molecular target for the chemotherapy of glioblastoma and other malignancies and that a therapeutic approach based on the ability of PKC inhibitors may be helpful in preventing invasion.

KEY WORDS: U-87 malignant glioma cell, Protein kinase C, Invasion.

Introduction

One of the most vexing properties of high-grade glioma is their ability to invade the surrounding normal brain tissue. Infiltrated cells often escape surgical resection and inevitably lead to tumor recurrence. The infiltrative nature of high grade glioma is responsible for much of the
morbidity and mortality associated with these tumours. The resultant poor prognosis and survival rate, with less than 10% of patients beyond 2 years underscore the need to further understand the mechanisms that underlie glioma invasion, and target cellular mechanisms that underlie tumor invasiveness.

Protein kinase C (PKC) is an important molecule in signal transduction that conveys signals generated by multiple ligand-receptor interaction to the nucleus. This family of serine/threonine kinases is derived from different PKC genes and from alternative splicing of a single transcript and consists of a single polypeptide chain divided into two domains: a regulatory domain at the NH2 terminus and a catalytic domain at the COOH terminus.

Both activation and down-regulation of PKC may be important in regulating cellular functions. PKC appears to be critical in many aspects of regulation of glioma cell biology.

The molecular mechanisms underlying tumor invasion remain poorly understood and the precise role of this enzyme in invasion is still subject to investigation. The purpose of this study was to more accurately assess the role of PKC in the invasion of human gliomas. U-87 cells were treated with the PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA) and inhibitor tamoxifen and hypericin and the resultant effects on the invasion of this cell line were examined.

Materials and Methods

1. Cell culture

Human glioma cell line (U87) was obtained from the American Type Culture Collection. The cells were cultured in a standard tissue culture incubator (37°C, 5% CO2, 95% air, 100% humidity). All tissue culture plastic articles were from Nunc (Roskilde, Denmark). Cells were regularly passaged by treatment with trypsin (0.05%) and were grown in a 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 (Grand Island Biological Company (Gibco), Grand Island, NY).

2. PKC modulation

For the modulation of PKC cells were treated with tamoxifen synthetic nonsteroidal antiestrogenic agent that inhibits protein kinase C by interfering with the activity of the catalytic subunit of the enzyme and hypericin a polycyclic aromatic dione that is a potent PKC inhibitor through interaction with the regulatory domain of the PKC enzyme. Cells were also treated with TPA which stimulates PKC and induces its rapid translocation from cytosol to plasma membrane.

3. PKC activity assay

The PKC activity assay was performed as in Chen et al. In brief, the treated cells were washed with Ca2+ and Mg2+ free phosphate buffered saline (PBS) three times and then lysed by sonication in 20 mM Tris-HCl (pH 7.4) buffer plus 2 mM EGTA, 0.5 mM EDTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 0.2 mM phenylmethyl-sulfonyl fluoride. Whole cell lysate was centrifuged at 300 x g for 12 min to remove debris. The Amersham PKC assay kit was used for the assay. The assay was performed according to manufacturer guidelines with an aliquot of supernatant (2 µg of protein) in the presence of Ca2+ and lipid and in the absence of Ca2+ or both Ca2+ and lipid to determine the activities of conventional (Ca2+ and lipid dependent) and novel (lipid dependent) PKCs.

4. Microculture tetrazolium (MTT) assay

The effects of tamoxifen, hypericin and TPA on cell growth were evaluated utilizing the MTT assay, in which the cell number was quantified by tetrazolium dye reduction. In brief, the cells were seeded into a 96-well cell culture plate at a density of 1000 cells/well in 200 µl volumes of 10% FBS-DMEM and incubated at 37°C. Following 24 h incubation, 100 µl medium with or without test drugs was aliquoted in the appropriate wells. After the 4-day incubation period, the number of viable cells was determined by measuring the bioreduction by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma, St. Louis, MO) this compound had been dissolved in sterile Hank’s balanced salt solution at 5 mg/ml and 50 µl of the solution had been added to each well. After 4 hour incubation at 37°C, all media were decanted, and 100 µl of mineral oil (Sigma, St. Louis, MO) was added to solubilize the MTT-formazan product. Light absorbance was determined by measuring optical density using a multiplate spectrophotometer (Thermo Molecular Devices Corp., CA) at 540 nm.

5. Matrigel artificial basement membrane invasion assay

Matrigel assay was performed as described by Albini et
al\textsuperscript{2} and Amar\textsuperscript{3}, and which we adapted to the multiwell Boyden chambers. Polycarboante filters (polyvinyl pyrrolidone-free, 13 mm diameter 12µm pores\textsuperscript{[6]} Poretics, Corp., Livermore, CA) were precoated with 1% gelatin and dried overnight and matrigel (Collaborative Biomedical, Bedford, MA, USA) whose constituents consist of collagen type IV, laminin, heparan sulfate proteoglycan, entactin and vitronectin was thawed and diluted to 0.5mg/ml with ice-cold double-distilled water. 100µl of this solution was spread over the entire surface of the gelatin-coated filter, yielding a 50µg barrier per filter and a matrigel density of 0.36 µg/mm\textsuperscript{2}. The matrigel was then dried at room temperature to a thin film under a laminar flow hood. Media conditioned by incubating log-phase NIH3T3 cells in serum-free medium supplemented with ascorbic acid (50µg/ml) for 24hrs was used as a source of chemo-attractant. The lower wells of the Boyden chamber were loaded with conditioned medium and the filters were placed coated-side up between the lower and upper wells. Exponentially growing malignant glioma cells were harvested with phosphate-buffered saline and suspended in modified Eagle’s medium with Earle’s balanced salt solution supplemented with 0.1% bovine serum albumin. 50,000 cells were then added to each upper well.

The chambers were placed in a humidified incubator at 37°C in 5% CO\textsubscript{2} for 5 hours, after which the filters were removed, air dried, and stained with the Diff-Quik staining system (Baxter Scientific product, McGaw Park, IL). The matrigel and the cells from the top surface were removed, air dried, and stained with the Diff-Quik staining system. The lipid-binding fluorescent carbocyanine dyes DiO (1,1’-dioctadecyl 3,3,3’,3’-tetramethylindo carbo-cyanine perchlorate) and DiI (3,3’-dioctadecyl oxacarbo-cyanine perchlorate) (Molecular Probes, Eugene, OR) are widely used in cell biology as tracers to study cell movement.

Briefly, mature brain aggregates with similar diameters of approximately 300µm were transferred individually into agar-coated 96-well culture dishes (Nunc Denmark). The volume of overlay medium was 1ml. Within 48 hours immature FRBAs formed. The FRBAs were transferred into individual wells and cultured for 21 days.

During this time, the cells aggregate and differentiate into normal adult rat brain elements in 3D spheroids, containing functional neurons, cortical lamination and functioning synapses, glial cells and an ependymal core, often with a microventricle\textsuperscript{9,10}.

Aggregates and spheroids with a diameter of 300±50µm were selected for the experiments.

Confrontation in culture of a tumor aggregate and a normal brain aggregate allowed the observation, quantitation and manipulation of the glioma invasion process \textit{in vitro}.

The confrontation pairs in culture were imaged after five days using continuous optical sections with a Bio-Rad
MRC 1024 laser scanning confocal microscope using PMT detectors and filter sets for rhodamine (DiI) and FITC (DiO). Pseudo-colored 8-bit planes were merged together and the distribution of DiI-labelled tumor cells within the DiO-labelled brain aggregate is plotted. Total amount of invasion is plotted against control.

**Results**

1. **Effect of tamoxifen, hypericin and TPA on the PKC activity of U-87 cells**

   To determine the level of PKC activity, each of the cell lines were assayed for Ca²⁺ and lipid-dependent protein kinase activity after 24 hour treatment, using partially purified cell extracts as described under "Experimental Procedures." PKC activity was assayed using histone III-S as the substrate for phosphorylation. As shown in Fig. 1, total PKC activity was dose dependently reduced 10%-

![Fig. 1. PKC activity in U-87 cell lines treated with tamoxifen(A), hypericin(B) and TPA(C). Total PKC activity was partially purified from each of the indicated cell lines and assayed in the presence of Ca²⁺ and lipid, using histone as a substrate. Total PKC activity was reduced dose dependently 10- 50% in cells treated with hypericin and 5- 20% in the tamoxifen treated group(p<0.05).](image)

![Fig. 2. U-87 cells treated with different concentrations of tamoxifen(A) and hypericin(B) showed a dose dependent inhibition of growth with the maximum inhibition occurring at a concentration greater than 500ng/ml in tamoxifen and 100ng/ml in hypericin in MTT assay(p< 0.01). When the cells were incubated with TPA(C) there was neither a definite cytotoxicity nor an increase in proliferating activity(p>0.122).](image)
50% in cells treated with hypericin and 5%-20% in the tamoxifen treated group (p<0.05).

2. Antiproliferative and cytotoxic effect of tamoxifen, hypericin and TPA

After the cell culture had been exposed for four days to tamoxifen, hypericin and TPA the number of viable cells was determined using MTT.

As shown in Fig. 2, treatment of U-87 cells with different concentrations of tamoxifen and hypericin caused a dose dependent inhibition of growth, with the maximum inhibition occurring at a concentration greater than 500ng/ml in tamoxifen and 100ng/ml in hypericin respectively (p<0.01).

When the cells were incubated with TPA for four days there was no definite cytotoxicity or increased proliferating activity (p<0.122).

3. Invasion of PKC U-87 cell through matrigel.

For the U-87 cell line which is treated with a PKC modulator for 24 hour, we calculated the invasiveness potential as being equal to the invasiveness observed in the matrigel assay minus the MTT cytotoxicity assay.

The tamoxifen treated PKC down regulated U-87 cell line exhibited significantly decreased invasive abilities, with dose dependent range of 29.5% to 41% when compared to the control cell line. The hypericin treated cell line also exhibited a decreased invasive ability range of 4% to 28% (p<0.05) (Fig. 3, 4). However, TPA treated cell lines exhibited no changes in invasive abilities (p=0.131).

4. Glioma invasion in tumor spheroid confrontation assay

In co-cultures of U-87 cell spheroids and brain aggregates, progressive invasion accompanied by destruction of the brain aggregate occurs. Glioblastomas showed both
diffuse and circumscribed infiltrative invasion accompanied often by cystic degeneration and massive destruction of the FRBAs (Fig. 5). Subjectively after 5 days of co-culture, about 30% of the initial brain volume was left in the control group. However, in the tamoxifen treated group, when the coculture was performed with U-87 cell line tumor spheroid, the remaining FRBAs volume was significantly increased by up to 70% in 100ng/ml and 90% in 500ng/ml, respectively when compared to the control. Hence, PKC appeared to increase the invasiveness of the tumor spheroid.

**Discussion**

Protein kinase C (PKC) is a phospholipid-dependent serine-threonine kinase that functions as an intermediary in the transduction of signals from the cell-surface to the level of the nucleus.

PKC is activated in the pathway of various growth factor
receptor-mediated proliferative signals, which include PDGF, EGF, and IGF. As a convergence point for growth factor signals, abnormalities in PKC activity have been found to contribute to the tumourigenesis of a wide variety of neoplasms. The apparent dependence of mitogenic signaling in astrocytes on PKC suggested to us that abnormalities of PKC may underlie the hyper-proliferative state of glioma cells. Many studies have reported that high grade gliomas are characterized by high levels of PKC activity and that inhibition of this second messenger system can dramatically reduce glioma growth in vitro.

Glioma in general, and more highly anaplastic gliomas in particular, infiltrate and spread great distances in the brain. Studies have shown that in the majority of cases tumor cells have migrated from the primary site of malignant gliomas by the time of diagnosis and are responsible for local recurrence and tumor progression.

The poor prognosis of patients with malignant gliomas is at least partially due to the invasive and by migration of single cells along vessels or white matter like corpus callosum.

Invasion is a multistep sequence of events requiring the integration of several factors. For a tumor to be invasive, the cells must penetrate the extracellular matrix and then move into the surrounding stroma. Specific types of cancers may express high levels of matrix metalloproteinase(MMP), serine or cystein protease. For example, human breast cancers express high levels of the aspartic protease cathepsin D and murine protease. For example, human breast cancers express high levels of the aspartic protease cathepsin D and murine protease. For example, human breast cancers express high levels of the aspartic protease cathepsin D and murine protease.

The process of glial tumor invasion has been studied in vitro for some time, but the molecular pathophysiology has not been examined. It is presumed that the processes of disordered adhesion, motility, and proteolysis are involved in glial tumor invasion. Extracellular matrix alterations have been described in model systems of glioma invasion as have motility factors in glioma cell lines. Based on these results and observations implicating PKC in the invasiveness of a variety of tumors, we decided to investigate the possibility that the abnormal high PKC activity in high-grade gliomas contributes to its invasive characteristics.

Several in vitro systems have been used to investigate different aspects of the invasion process. Commonly used methods of assessing brain tumor invasiveness in vitro involve measuring migration across an artificial basement membrane of brain extracellular matrix(ECM) components or through a chemotaxis chamber filter. Another model for measuring tumor invasiveness is confrontation assay with aggregates of dissociated fetal brain cells or with fragments of embryonic tissues such as heart muscle.

To obtain satisfactory results with this invasion study, we used both methods with a PKC modulator. Results demonstrate that inhibition of PKC can reduce the ability of U-87 cells to invade both through the artificial membrane matrigel coated filter and when confronted to normal fetal rat brain aggregates.

In addition, we couldn’t get both high PKC activity and increased invasion potential in TPA treated cell. This may due to the fact that TPA stimulates PKC via inducing rapid translocation of PKC from cytosol to plasma membrane and they disappear rapidly within few minutes.

Our data supports the hypothesis that PKC-mediated pathways are in control of the invasion property of tumor. The phospholipid- and calcium- dependent PKC is found ubiquitously in the developing brain, and in the normal adult brain it is expressed at high levels in the neurons.

Several aspects of glioma biology are similar to the properties of fetal glial cells. Glioma cells and fetal astrocytes were found to have a similar migration into hydrated collagen I gels.

Moreover, the production of proteases characteristic of migratory cells was similar in gliomas and fetal astrocytes. This can explain why we sometimes see fetal brain cells inside of the tumor spheroid. Recently Uhm et al, demonstrated that the invasiveness of human glioma cells in vitro correlates with the activity of MMP-2, which in turn, may be regulated by signal transduction through PKC. The treatment of highly invasive glioma cells with calphostin C, a selective inhibitor of PKC, led to a decrease in the MMP-2 activity with a concomitant reduction in glioma invasiveness. The researcher assumed that PKC modulates MMP-2 activity and invasiveness of gliomas through the formation of the Jun/Fos transcription factor. However, MMP-2 cannot be induced in this manner, because its gene lacks the promoter sequence elements AP-1 required for induction by Jun/Fos. Therefore, PKC regulation of MMP-2 activity is likely to be post-transcriptional and/or post-translational. Based on this data, the increased MMP-2 activity in human gliomas may be secondary to a PKC-mediated stimulation of the membrane-type MMP(MT-MMP), the enzyme which proteolytically activates MMP-2. In addition to its effect on MT-MMP and MMP-2 activity, PKC may...
induced by PKC activation. Furthermore, cathepsin B, a cysteine protease, has been shown to be required to proteolytically activate most MMPs. Additionally, cathepsin B, a cysteine protease, has been shown to be induced by PKC activation.

While the presence of these proteases has been demonstrated in gliomas, the role of PKC in modulating their expression pattern is not known at this time.

Promising results in vitro have led to studies in patients using pharmacological agents that demonstrate anti-PKC effects (tamoxifen) and which have demonstrated clinical and radiological improvements in a limited subset of glioma patients. Furthermore, the use of antisense oligonucleotides that are complementary to the mRNAs encoding specific PKC isoforms have also demonstrated striking results against gliomas in animal models.

This is an important consideration with respect to potential toxicity in vivo, especially since various PKC isoforms are expressed in many normal cells of the nervous system, including astrocytes, oligodendrocytes and neurons. As to which PKC isoform mediates invasiveness is not well known. In this study we found evidence that PKC is involved in glioma cell invasion and such information may be invaluable in the design of more selective therapeutics.

References

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교모세포종 세포주 U-87에서 세포내 PKC 농도와 종양침습성과의 상관 관계

지 천·조경근·이정진·박성환·조정기·강준기·최창락

= 국문조목 =

교모세포종 세포주 U-87에서 세포내 PKC 농도와 종양침습성과의 상관 관계를 연구하기 위한 실험을 수행하였다. 실험은 U-87 세포주에서 PKC 농도에 따른 종양침습성과의 상관 관계를 조사하였다. 실험결과, PKC 농도가 증가함에 따라 종양침습성은 증가하는 경향을 보였으며, 이러한 결과는 PKC가 세포 내의 종양침습성과 관련이 있을음을 시사한다. 또한, tamoxifen과 hypericin의 PKC 억제 효과가 종양침습성과 상관관계가 있는 것으로 나타났다.

결론적으로, U-87 세포주에서 PKC의 농도는 종양침습성과의 상관 관계가 있는 것으로 보이며, tamoxifen과 hypericin의 PKC 억제 효과가 종양침습성과 상관관계가 있는 것으로 보인다.