Reduction of protein kinase C delta attenuates tenascin-C stimulated glioma invasion in three-dimensional matrix

Susobhan Sarkar and V. Wee Yong
Hotchkiss Brain Institute and the Departments of Clinical Neurosciences and Oncology, University of Calgary, Calgary, Alberta, Canada

Introduction

The dismal outcome of patients with malignant gliomas is attributed to the highly invasive nature of tumor cells into surrounding normal brain tissue leading to new areas of multifoci growth. The invasiveness of glioma cells is mediated in part by their interaction with the extracellular matrix. We have reported that in a three-dimensional matrix of type 1 collagen (3D-CL) gel, the extracellular matrix protein tenascin-C (TN) increased the invasiveness of glioma cells through the downstream production of matrix metalloproteinase (MMP)-12. In the present study, we have investigated the signaling mechanisms involved in the TN-stimulated glioma invasiveness. We found that the pan protein kinase C (PKC) inhibitor, bisindolylmaleimide I, decreased TN-enhanced glioma invasion in 3D-CL. Calphostin C, an inhibitor of conventional and novel PKC isoforms, and the relatively selective PKCδ inhibitor rottlerin decreased TN-stimulated glioma invasiveness in a concentration- and time-dependent manner. These findings of the possible involvement of PKCδ was supported by its translocation from the cytosol to membrane fraction in 3D-CL gel supplemented with TN as detected by western blot assays and immunofluorescence microscopy and by elevation of PKCδ enzyme activity. Moreover, pharmacological blockade of PKCδ decreased MMP-12 levels and glioma invasiveness. Finally, small interfering RNA to PKCδ reduced TN-stimulated glioma invasiveness concurrent with decreased MMP-12 production. Our results implicate PKCδ as a therapeutic target to reduce MMP-12 expression and glioma invasiveness when tumor cells are stimulated by the TN-enhanced glioma microenvironment.

Materials and methods

Cell culture

The U178 or U251 human glioma cell line was originally obtained from American Type Culture Collection (Manassas, VA) and maintained in minimum essential medium supplemented with 10% fetal bovine serum. For three-dimensional (3D) cell culture, we prepared type 1 collagen (CL) gel on ice as per manufacturer’s instruction (Chemicon, Temecula, CA). Briefly, for preparing 500 μl of CL gel, 400 μl of cold CL solution was mixed with 100 μl of cold 5× Dulbecco’s modified Eagle’s medium and the mixture was pH neutralized. Unless otherwise stated, 1 million glioma cells were added to 500 μl of CL solution, supplemented with or without test compounds (such as TN or PKC inhibitors). The plate was kept immediately at 37°C for 1 h for polymerization, trapping cells within the CL. After the CL gel had formed and became a 3D-CL matrix, it was covered with culture media. CL was used to generate a 3D matrix because it readily formed the gel when warmed to 37°C and into which glioma cell could be embedded. Importantly, CL is an ECM molecule expressed around glioma cells in situ (31,32).

Invasion assays

Glioma cells were trypsinized with 0.25% trypsin-ethylenediaminetetraacetic acid and were suspended in CL solution as described above. Test molecules including TN (10 μg/ml) were added into the CL solution. The TN (CC065, Chemicon) used in the present study was human TN-C protein with a molecular weight around 280–300 kDa. Following pipetting to mix cells and test molecules into the CL solution, 70 μl (140 000) of cells were distributed onto the center of the top compartment of Transwell migration chambers (Corning, 3422, polycarbonate membrane, 24-well format, 8 μm pore size (Corning, NY)) (18). Following polymerization of the CL I droplet, 100 μl of Dulbecco’s modified Eagle’s medium/F-12 medium with N2 supplement was added to the upper chamber and 1 ml of 10% fetal bovine serum-containing medium was applied to the lower well. Cells were then allowed to invade out of the 3D-CL matrix, across the membrane, at 37°C for 24 h. Non-invasive cells were removed from the top compartment of the Transwell with a cotton swab and the invasive cells present on the underside of the membrane were fixed and stained with hematoxylin. The number of invasive cells was counted per field (×40 microscope objective) from four random fields of each membrane.
When PKC inhibitors were used, they were added within the gel and also in the medium in the upper and lower chambers of the Transwell. The concentra-
tion of inhibitors described in the Results refers to the final concentration
within the gel and culture medium.

Subcellular fractionation and translocation of PKC isoforms in 3D-CL matrix
Glioma cells (5 × 10^6) were suspended into 500 μl of cold CL gel solution and
plated onto a well of a 24-well plate. Cell–gel solution was supplemented with
or without test molecules (e.g. TN) and was allowed to polymerize at 37°C as
described earlier. The polymerized gel was then covered with 300 μl of Dulbecco's modified Eagle's medium/2% (N2 supplemented) FBS. Inhib-
itors of PKC were added to the medium as well as within the gel; the PKC
activator, phorbol-12,13-myristate acetate (PMA), was similarly utilized. At
desired time points, media was removed and 500 μl of digitonin lysis buffer (1 M Tris–HCl, pH 7.5, 100 mM ethyleneglycol-bis(aminoethyl ether)-
tetraacetic acid, 100 mM ethylenediaminetetraacetic acid, 10 mg/ml digitonin,
100 mM NaF; 100 mM sodium orthovanadate; protease inhibitors were added to
the buffer immediately before use) was added to the gel. With repeated pipetting,
the CL gel disintegrated and the resulting cloudy solution was homogenized for
10 s in a homogenizer. Cells were further lysed by sitting on ice for 10 min. Next,
the cell lysate was ultracentrifuged at 100 000g for 45 min at 4°C. The super-
natant was collected as the cytosolic fraction of cells. The pellets was suspended
in 60 μl of digitonin–Triton X-100 lysis buffer (digitonin lysis buffer supplemented
with 1% Triton X-100), incubated on ice for 30 min, centrifuged for 10 min at
10 000g at 4°C and the resulting supernatant was collected as the particulate
(membrane) fraction. The total protein concentration of each fraction was
determined using the Bio-Rad protein assay. Equal amounts of protein
(50 μg) from each fraction were resolved on 10% sodium dodecyl sulfate–
polyacrylamide gel electrophoresis for western blot analysis.

PKC activity assay following immunoprecipitation
Equal amount of total cellular protein (500 μg/ml) from each subcellular
fraction was incubated with 20 μl (4 μg) of PKCδ antibody (C-17-SC 213,
Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C. Twenty microliters
of protein A/G plus-Agarose beads were added and incubated at 4°C for 1 h at
overnight. The immunoprecipitate was collected by centrifugation
of protein A/G plus-Agarose beads were added and incubated at 4°C for 1 h at
overnight. The immunoprecipitate was collected by centrifugation.

Immunofluorescence for PKC

Triton X-100 in PBS was then added to permeabilize cells. A primary rabbit
peptide. The absorbance is directly proportional to the PKC activity.

Ontario, Canada). Following color development, the absorbance was measured at
312 nm. Gelatin Zymography

Gelatin zymography to determine the amounts of pro- and active-MMP-2 has
been described elsewhere (8).

For transfection with siRNA, glioma cells were plated in 24-well plates and
were incubated with 33 nM siRNA and lipofectamine (Invitrogen), as de-
predicted previously for RNAi experiments (18). After 48 h, cells were harvested
for invasion assay or MMP-12 detection as described above.

Statistics

The one-way analysis of variance test with post hoc Tukey–Kramer multiple
comparisons was used.

Results

In 3D-CL matrix, the TN-stimulated glioma invasiveness is reduced by
inhibitors of PKC

In previous work (18), we found that when encased in a 3D-CL matrix
supplemented with TN, glioma cells increased their movement out of
the CL gel onto the other side of a filter where their numbers could be
quantiﬁed as an index of glioma invasiveness. To determine whether or not the PKC pathway was involved in TN-stimulated glioma
invasiveness in 3D-CL matrix, we employed pan-specific (bisindolyl-
maleimide) or conventional and novel PKC-selective (calphostin C)
inhibitors. We found that the number of U178 glioma cells that
invaded across the TN-supplemented CL matrix was reduced by both
inhibitors (Figure 1), which is also the case for U251 glioma cells
(Figure 2). This reduction occurred at concentrations known to inhibit PKC and was
due to toxicity of bisindolylmaleimide or calphostin C as deter-
mined through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide assay (data not shown). When the U178 and U251 lines were
allowed to invade across the 3D-CL matrix in absence of TN, glioma
invasiveness was not altered in presence of inhibitors (supplementary
Figure 2 is available at Carcinogenesis Online). These data suggest
that PKC isoforms were engaged in glioma invasiveness in a 3D-CL
matrix only when TN was present.

PKCδ does not have a major role in TN-stimulated glioma invasiveness

From the observation that bisindolylmaleimide or calphostin C de-
creased TN-stimulated glioma invasiveness in 3D-CL, we next sought to
determine the involvement of particular PKC isoforms. We first
employed G0 6976, described as a selective inhibitor for PKCδ (28).
Although the addition of G0 6976 into the CL + TN 3D matrix
decreased invasiveness of U178 glioma cells, the apparent IC_{50} (over
100 nM) to reduce invasiveness was beyond its reported IC_{50} value
(2.3 nM) for inhibiting conventional PKC isozymes (Figure 2A). Similar
result was also observed with U251 line (supplementary Figure 3 is
available at Carcinogenesis Online). These results imply the minor
involvement of PKCδ in TN-stimulated glioma invasiveness, which
was supported by the observation that TN did not translocate PKCδ
from the cytosol to the particulate (membrane) fraction of cells
(Figure 2B). Translocation of PKC isoforms is an index of their
activation and the positive control PMA translocated PKCδ from
the cytosolic to the particulate fraction after 1 h (Figure 2B).

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PKC d appears important for TN-stimulated glioma invasiveness in 3D-CL matrix. The result that PKC a is not a major mediator of TN-stimulated glioma invasiveness, coupled with the absence of other conventional PKC isoforms in our glioma lines (33,34), led us to examine novel PKC isoforms. We found that TN did not translocate PKC e either after 1 or 3 h (data not shown). In contrast, PKC d was prominently translocated in cell fractionation studies by TN, to the extent achieved by the addition of bisindolylmaleimide or calphostin C to TN-supplemented CL gels (CL + TN) decreased invasiveness (**P < 0.001 compared with CL + TN) (A and B). Columns represent the mean ± SEM of four analyses and the result was reproduced across another set of experiment. Panel (C) depicts pictorially the effect of calphostin C on glioma invasiveness after 24 h (original magnification ×400).

Fig. 1. TN-stimulated glioma invasiveness is reduced by PKC inhibitors. Tenascin-C (henceforth denoted as TN in this and all figures) that is embedded within CL gels increased invasiveness of U178 glioma cells (A and B); **P < 0.001 of CL + TN compared with CL alone. The addition of bisindolylmaleimide or calphostin C to TN-supplemented CL gels (CL + TN) decreased invasiveness (***P < 0.001 compared with CL + TN) (A and B). Columns represent the mean ± SEM of four analyses and the result was reproduced across another set of experiment. Panel (C) depicts pictorially the effect of calphostin C on glioma invasiveness after 24 h (original magnification ×400).

Fig. 2. PKCα plays a minor role in TN-stimulated glioma invasion. The TN-increased invasiveness of U178 glioma cells (**P < 0.001 of CL + TN compared with CL alone) was attenuated in a concentration-dependent manner by the PKCα selective inhibitor, G0 6976 (A). However, the effective concentrations of G0 6976 to reduce invasion were mostly beyond its IC50 value of 2.3 nM (28) to inhibit PKCα enzyme activity; ***P < 0.001 compared with CL + TN. Columns are mean ± SEM of four wells and the result was reproduced in another set of experiment. In panels (B), western blot analysis was conducted of the subcellular distribution of PKCα between cytosolic and membrane particulate (P) fractions of U178 glioma cells following phorbol ester (PMA, positive control) or TN treatment of cells grown in CL gel. Protein extract collected after 1 or 3 h following PMA or TN treatment were used. Equal amount of proteins were loaded in each well. While PKCα was translocated following PMA treatment, TN did not produce translocation of PKCα after 1 h and minimally after 3 h. Cell lysate obtained at 0 h served as additional control of the basal level of PKCα in cells. Actin was used as loading control for respective cytosolic or particulate fractions. This experiment was reproduced twice.

**PKCα appears important for TN-stimulated glioma invasiveness in 3D-CL matrix**

The result that PKCα is not a major mediator of TN-stimulated glioma invasiveness, coupled with the absence of other conventional PKC isoforms in our glioma lines (33,34), led us to examine novel PKC isoforms. We found that TN did not translocate PKCα either after 1 or 3 h (data not shown). In contrast, PKCδ was prominently translocated in cell fractionation studies by TN, to the extent achieved by the
potent PKC stimulator, PMA (Figure 3A). The involvement of PKC was further supported by the increase in PKC enzymatic activity in response to TN stimulation in the 3D-CL matrix (Figure 3B).

We next performed immunofluorescence analyses to investigate whether the translocation of PKC could be detected at the level of individual cells. Figure 3C shows that while PKC immunofluorescence was diffusely distributed across cells when grown in a CL matrix, the addition of TN resulted in accumulation of PKC at the plasma membrane, in a manner similar to that found when PKC of cells were activated by the positive control PMA.

A selective inhibitor of PKC decreases TN-stimulated glioma invasion and MMP-12 expression

To further investigate the involvement of PKC in TN-stimulated glioma invasiveness, we employed rottlerin, a relatively selective inhibitor of PKC (35). The addition of rottlerin to the CL + TN 3D matrix significantly decreased U178 glioma cell invasiveness in a concentration-dependent manner (Figure 4A) and time-dependent manner (Figure 4B) without any obvious toxicity (data not shown). Rottlerin also decreased the invasiveness of U251 glioma cells (supplementary Figure 4 is available at Cancerogenesis Online).

We addressed whether inhibition of PKC would alter MMP-12 expression. Figure 5A shows that the addition of rottlerin to the CL + TN matrix reduced TN-stimulated MMP-12 expression as detected in either cell-conditioned media or from immunoprecipitates. These results of PKC inhibition provided an important link between TN exposure and MMP-12 production downstream.

siRNA experiments further validate that reduction of PKC attenuates glioma invasiveness and MMP-12 expression

The likelihood that TN-mediated glioma invasiveness involves PKC was next examined by RNA interference. Figure 5B shows that the three different siRNAs to PKC reduced PKC protein level in glioma cells; in contrast, PKCα and ε levels were not affected (data not shown). In addition, knock down of PKC with siRNA reduced MMP-12 expression. Concordantly, glioma invasiveness promoted by TN in 3D-CL matrix was attenuated by PKC RNAi treatment (Figure 5C).
Discussion

An extensive deposit of various ECM molecules surrounds glioma cells in situ, where they regulate the behavior of transformed and non-transformed cells alike. Among the various ECM molecules, TN appears to be very important for glioma pathophysiology, as its expression level correlates inversely with prognosis and survival of patients (9–12). In a series of 62 cases, gliomas with diffuse intercellular TN immunoreactivity showed higher proliferation index than those with focal and limited expression (36). A monoclonal antibody to TN that was labeled with 131I slowed tumor growth and prolonged survival in rodents implanted in the flank or brain with human glioma cells (37,38). The 131I-labeled anti-TN antibody has since been tested in a pilot trial in patients with malignant glioma where encouraging survival results have been obtained (39). Other means to target TN in gliomas, such as by the use of siRNA, are being tested (40).

In a 2D culture system, TN was found to be very permissive for the migration of glioma cells (16). Of several ECM proteins found in gliomas and tested for their ability to induce the haptotaxis of human neural stem cells in 2D, in work that attempts to elucidate interactions in the glioma microenvironment, TN was found to be the most potent (41). Taking into consideration that a 3D matrix is a more physiological environment to evaluate cell behavior, we have examined and reported that TN encapsulated within 3D-CL promoted the invasiveness of glioma cells out of the 3D constraint through an MMP-12-dependent mechanism (18). To characterize this further, the present study has attempted to determine the signal transduction pathways through which TN in 3D-CL regulates MMP-12 expression and glioma invasiveness. Such investigations may provide additional targets to inhibit TN-mediated glioma pathophysiology.

We have implicated the PKC system, particularly PKCδ, in the TN-simulated MMP-12 production and glioma invasiveness. This conclusion is derived from biochemical (Figure 3), pharmacological (Figure 4) and siRNA (Figure 5) experiments. From pharmacological or translocation experiments (Figures 1 and 2, and S. Sarkar, V.W.Yong, unpublished data), we found no evidence for the involvement of PKCα or PKCε in TN-mediated glioma invasiveness in the 3D context. Our findings raise the possibility that glioma invasiveness promoted by TN may be reduced not only by MMP-12 or TN inhibitors but also by agents that attenuate PKCδ level or activity.

The PKC enzyme system has been implicated by several groups including our own in various aspects of glioma biology, such as invasiveness, proliferation, MMP expression, apoptosis and survival (42,43). In this regard, it appears that depending on the microenvironment that it encounters, a glioma cell may utilize different PKC isoforms to achieve particular functions. For example, in a 2D format that is not constrained by ECM proteins, PKCε appears to regulate migratory behavior (33,34). In contrast, in the present study where glioma cells are encased within a 3D-CL matrix supplemented with TN, we found no evidence for the involvement of PKCε; rather, PKCδ was prominently engaged. Moreover, while PKCα has been implicated in glioma invasiveness in a 2D context (44), we did not find evidence for its role in regulating invasiveness in the TN–CL 3D matrix. Given the close correlation between glioma pathophysiology and TN expression in vivo, we would suggest that it is more critical to target PKCδ rather than other PKC isoforms against glioma invasiveness.

The complexity of glioma cells engaging specific PKC isoform depending on the microenvironment has also been noted for other tumors, probably because of the many different roles and mode of regulation of PKC isozymes that vary among many different cell types (45). Nonetheless, PKCδ has been implicated in invasiveness of other tumor types, such as in breast cancer (46,47). Clearly, the role of the different isoforms of PKC in glioma cells, and how they regulate the many different pathways involved in malignant transformation (48),

![Fig. 4. TN-stimulated glioma invasion is attenuated by the PKCδ inhibitor, rottlerin. The increased invasiveness of U178 glioma cells in CL + TN gel (**P < 0.001 of CL + TN compared with CL alone) was reduced by rottlerin in a concentration-dependent manner (A); ***P < 0.001 compared with CL + TN. Moreover, the increase in invasiveness from 3 to 24 h of seeding of cells into the CL matrix promoted by TN (**P < 0.001 compared with CL alone) was attenuated by 5 μM rottlerin (B). Columns represent the mean ± SEM of four analyses and the result was reproduced twice.](https://academic.oup.com/carcin/article-abstract/31/2/311/2477346)
deserves further attention. In this regard, it is encouraging that PKC inhibitors continue to be the subject of clinical trials in gliomas (49).

In summary, our results implicate PKCδ in the mechanism of TN-promoted MMP-12 expression and glioma invasiveness. The results are potentially relevant to glioma therapeutics since TN is an ECM protein very prominently associated with glioma pathophysiology. Several questions remain, including the cell surface receptors that transduce signals from TN to intracellular PKCδ, and the precise mechanisms by which PKCδ links to MMP-12 generation. These questions are the subject of future studies that aim to improve the prognosis of patients with malignant gliomas.

Supplementary material
Supplementary Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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References

Fig. 5. Reduction of PKCδ by pharmacologic inhibition (rottlerin) or siRNA attenuates TN-stimulated glioma invasion and MMP-12 production. In panel (A), the addition of rottlerin (5 μM) within CL + TN gel decreased TN-stimulated MMP-12 expression by glioma cells. Decreased level of pro- and/or active form of MMP-12 either from conditioned media (upper panel) or from cell immunoprecipitate (lower panel) was detected following rottlerin treatment. The pro- and active forms of MMP-2 present in cell-conditioned media were evaluated by gelatin zymography to provide evidence of equal loading between the groups since MMP-2 was not found to be altered by TN treatment in previous reports (18). In panel (B), three siRNAs targeted toward different regions of PKCδ reduced expression of PKCδ as detected by western blot analyses; in contrast, PKCα or ε levels were not reduced (data not shown). In addition, decreased level of MMP-12 was detected in the same extracts (B). glioblastoma multiforme refers to a resected human glioma homogenate. When PKCδ siRNA-treated cells were allowed to invade through TN-supplemented collagen gels (CL + TN), their invasiveness was attenuated compared with control siRNA-treated cells (C); **P < 0.01, ***P < 0.001 compared with control siRNA.
Tenascin-C mediated glioma invasion and PKC


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