Laminin-induced PC-12 cell differentiation is inhibited following laser inactivation of cellular prion protein

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Abstract Prions, the etiological agents for infectious degenerative encephalopathies, act by inducing structural modifications in the cellular prion protein (PrPc). Recently, we demonstrated that PrPc binds laminin (LN) and that this interaction is important for the neuritogenesis of cultured hippocampal neurons. Here we have used the PC-12 cell model to explore the biological role of LN–PrPc interaction. Antibodies against PrPc inhibit cell adhesion to LN-coated culture plaques. Furthermore, chromophore-assisted laser inactivation of cell surface PrPc perturbs LN-induced differentiation and promotes retraction of mature neurites. These results point out to the importance of PrPc as a cell surface ligand for LN.

Key words: Cellular prion protein; Laminin; Extracellular matrix; Neurite outgrowth; PC-12 cell; Cell differentiation

1. Introduction

Prions are unprecedented infectious pathogens that cause a group of invariably fatal neurodegenerative diseases by an entirely novel mechanism, which involves posttranslational structural modification of the cellular prion protein (PrPc), leading to the deposition of an insoluble and protease-resistant isoform (PrPsc) within the central nervous system [1,2].

The extracellular matrix glycoprotein laminin (LN) plays a major role in neuronal differentiation, characterized by neurite formation and extension [3], migration [4,5] and regeneration [6]. In the central nervous system, besides the basement membranes of brain vasculature, LN is expressed by hippocampal neurons [7,8]. We have recently demonstrated that PrPc binds LN in a high-affinity, specific and saturable fashion [9]. This interaction seems to have a role in the neuritogenesis of rat and mouse hippocampal neurons. LN immunolabeling disappears from the rat hippocampus after kainic acid injection; this event precedes neuronal death and can be blocked by tPA deficiency or infusion of plasmin inhibitor, suggesting that LN–neuron interactions prevent cell death [8]. Recent study [10] has shown that PrPc prevents serum deprivation-dependent apoptosis of neurons in culture and suggested that PrPc might be similarly involved in neurite extension by these cells. We on the other hand have observed that PrPc-null mice, in which the *PRNP* gene has been ablated, are far more sensitive to kainic acid and three other distinct seizure-inducing models [11]. Moreover, we also demonstrated decreased LN-induced neuritogenesis in cultured PrP-null mice hippocampal neurons [9].

Chromophore-assisted laser inactivation (CALI) of protein function has been successfully applied in different models [12–17] with a high degree of spatial and temporal resolution. This technique uses laser energy to inactivate a single protein following its binding with antibodies labeled with the chromophore malachite green (MG), which generates short lifetime hydroxyl free radicals [13].

Herein, we provide evidence that specific antibodies against PrPc inhibit PC-12 cell adhesion to LN. Using the same antibodies coupled with MG, we have observed inhibition of LN-mediated PC-12 cell differentiation and retraction of pre-extended LN-induced neurites following laser application.

2. Materials and methods

2.1. Cell culture

PC-12 cells (kindly provided by Dr. Paulo Lee Ho, Instituto Butantan, SP, Brazil) were primed during 5 days with 50 ng/ml NGF-β (Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) plus 10% fetal calf serum (FCS) (Cultilab, Campinas, Brazil), harvested, resuspended in DMEM and used for the large-scale CALI assays (these cells were considered non-differentiated due to their round morphology under phase-contrast microscopy). For the micro-scale CALI analysis, cells were primed as described above and differentiated for 24–48 h on slide coverslips coated with poly-L-lysine (Sigma) and EHS-purified LN [18] (10 μg/ml in phosphate-buffered saline (PBS), overnight at 4°C) in DMEM 2.5% FCS containing 50 ng/ml NGF-β.

2.2. Immunofluorescence

PC-12 cells were differentiated as described above on glass chamber slides (Nunc, Naperville, IL, USA). Living cells were incubated for 1 h at 4°C with anti-GST-PrPc (mouse recombinant protein) or anti-GST sera [19] (diluted 1:80 in DMEM), washed three times with PBS and fixed in 3.7% paraformaldehyde solution during 30 min. Cells were washed as described above and then incubated with anti-rabbit FITC-conjugated IgG (Sigma) diluted 1:80 in PBS for 1 h at room temperature. After another washing step, cells were mounted in glycerol, observed and documented with a fluorescence microscope (Nikon SMZ-10).

2.3. Cell adhesion to LN-coated plates

Non-differentiated PC-12 cells were harvested and incubated for 1 h at 4°C with sera against GST, GST-PrPc, α1, α3 and β1 integrin

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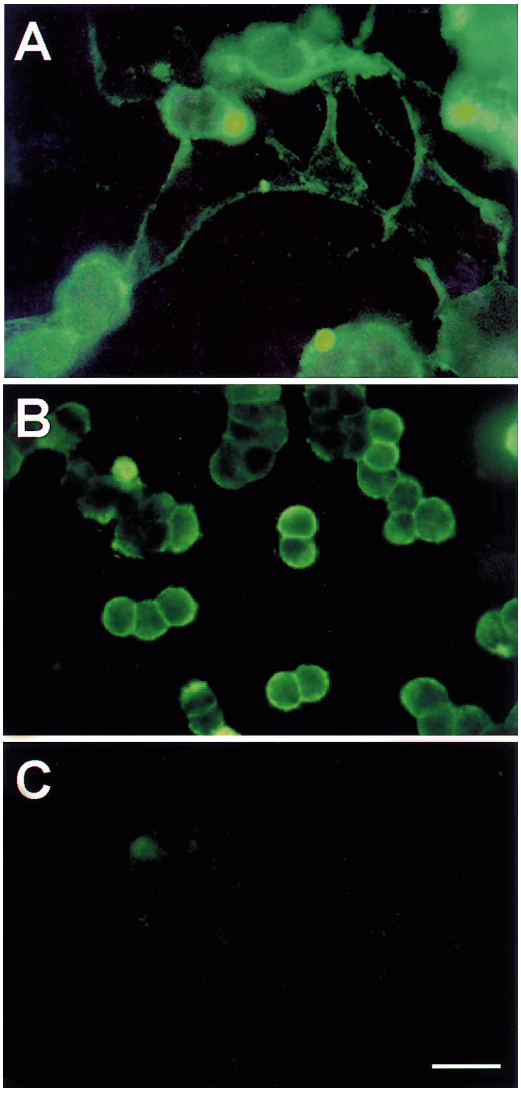

Fig. 1. Presence of PrPc protein on cell surfaces revealed by immunofluorescence reactions using anti-GST-PrPc antibodies. LN-differentiated (A) and non-differentiated (B) PC-12 cells. There was no immunolabeling with anti-GST serum (C). Scale bar, 25 μm.

chains (Chemicon, Temecula, CA, USA) or normal rabbit serum (diluted 1:100 in DMEM). After plating on LN-treated 96-well culture plaques (Costar, Cambridge, MA, USA) and incubation for 1 h at 37°C, cells were uniformly washed 7–10 times with PBS, the remaining cells were fixed as described above, stained with toluidine blue (Sigma) and washed several times with distilled water. Cells were then incubated for 15 min at 37°C with 100 μl of 1% SDS (Gibco BRL) solution and the optical density of the supernatants analyzed with the aid of an enzyme-linked immunosorbent assay reader at 580 nm (Bio-Rad Benchmark Microplate Reader).

2.4. CALI

The CALI system uses laser energy to inactivate a target protein following its binding with specific antibodies labeled with MG. Therefore, in our model, the interaction between PrPc at the cell surface and the LN-coated culture plate is abrogated.

Large-scale CALI [14,15]: a Quanta Ray GCR-11 laser (Spectra Physics Corp., Mountain View, CA, USA) was used to drive a dye laser containing the fluorescent laser dye DCM (Exciton Corp., Dayton, OH, USA) to generate a 620 nm pulsed laser beam with a pulse width of 3.5 ns at a frequency of 10 Hz and a peak pulse energy of 15 mJ. PC-12 cells were incubated during 1 h at 4°C with MG-labeled anti-PrPc antibodies: anti-GST-PrPc and anti-prion N-10 (kindly provided by Dr. Stanley Prusiner, University of California, San Francisco, CA, USA) or purified rabbit IgG (Sigma). After the incubation, the cells (3.5×10^4/well) were pelleted and washed to remove unbound MG-labeled antibodies, resuspended and transferred into 96-well prong plates (Intermed, Denmark) for CALI treatment. Wells were then subjected to 1 min of continuous laser pulsing at 15 mJ/pulse. After laser treatment, PC-12 cells were pooled, pelleted, resuspended in 300 μl of DMEM 2.5% FCS and plated on 24-well plates (Costar) previously coated with poly-L-lysine (Sigma) plus LN. After incubations of 8 h at 37°C and 5% CO_2, the cells were fixed as described above and triplicate wells analyzed by phase-contrast microscopy. The number of cells with neurites was counted in 15 fields per well. Micro-scale CALI [16,17]: 24–48 h after plated over the LN substrate, differentiated PC-12 cells were kept at 37°C at a phase-contrast microscope with the aid of a stage incubator during the micro-scale CALI manipulations. Laser energy for micro-scale CALI was comparable to that used in large-scale experiments (nitrogen-driven dye laser, VSL-337, Laser Science Co., Newton, MA, USA). Growth cone behavior was observed by time-lapse video microscopy. Image analysis was aided by the NIH Scion Image Software. The results represent the behavior of nine anti-PrPc-treated and nine normal IgG-treated PC-12 cells.

3. Results and discussion

The binding between LN and PrPc was recently demonstrated by our group using the GST-PrPc fusion protein [9]. Since both LN and PrPc seem to mediate neuronal survival [8,10] and are expressed in the hippocampus [7,8,20,21], we decided to further investigate the possibility that PrPc was one of the neuronal LN receptors.

LN has been found to promote cell adhesion in vitro and a number of cell surface receptor proteins such as integrins and other non-integrin LN receptors [22,23] have been identified as mediators of this activity. Here we used the PC-12 cell line, a well established model of neuronal differentiation [24,25] that expresses PrPc on its surface, both in non-differentiated and LN-differentiated state (Fig. 1), to address the biological function of LN–PrPc interaction. Treatment of PC-12 cells with anti-GST-PrPc antiserum, before plating the cells on

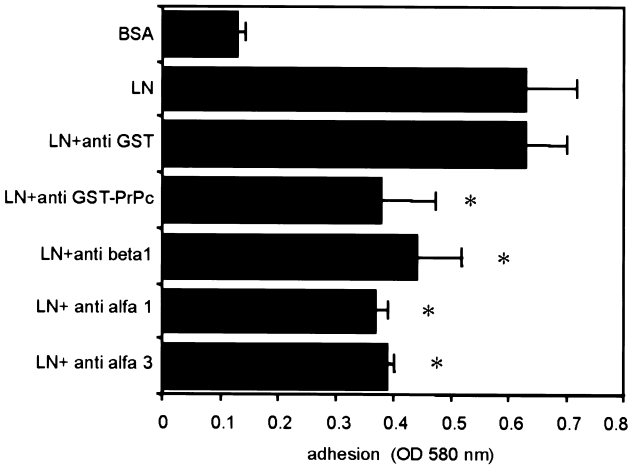

Fig. 2. Antibodies against PrPc (anti-GST-PrPc), as well as anti-α1, -α3 and -β1 integrin chains, significantly inhibit adhesion of PC-12 cells on the LN substrate. Anti-GST serum (anti-GST) in the culture medium and bovine serum albumin (BSA) as substrate for cell adhesion were used as negative controls. LN, control without preincubation with antibodies. The results represent the average and standard deviations of three independent experiments ($^*P < 0.05$; Mann–Whitney test for unpaired samples).

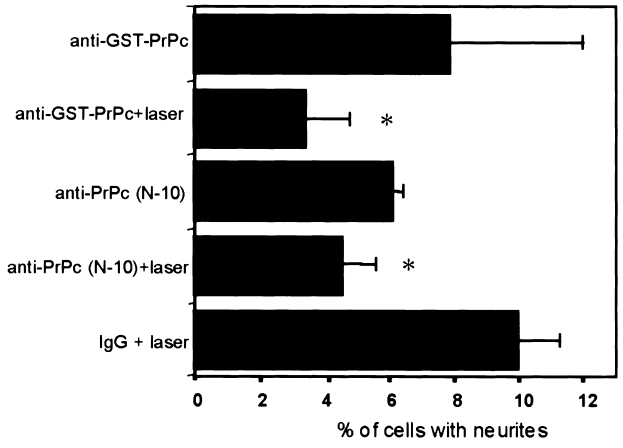

Fig. 3. Large-scale CALI. PC-12 cells were incubated with MG-labeled IgG purified antibodies anti-GST-PrPc; anti-PrPc (N-10) or non-immune rabbit IgG (IgG), submitted to laser treatment and plated on LN substrate. After an incubation period of 8 h, both anti-PrPc antisera significantly inhibited LN-induced neurite outgrowth. Results represent the average and standard deviations of three independent experiments (*$P < 0.001$; Mann–Whitney test for unpaired samples).

LN-coated culture plates, inhibits cell adhesion by 30% ($P < 0.05$) (Fig. 2). Serum raised against the GST protein contained in the fusion protein GST-PrPc did not cause any effect when compared to control without antibody treatment (LN). Positive controls using sera against the integrin chains α1, α3 and β1 also showed a 30–35% inhibition ($P < 0.05$) (Fig. 2), similar to what has previously been reported [26]. These findings suggest that PrPc may have a role in cell adhesion to LN.

CALI is a method of protein ablation induced by photochemical damage directed by MG-conjugated antibodies, without any effect on other cellular components or living cells by itself [16]. This technique avoids compensatory mechanisms that may be elicited in genetically modified animals or cells. To perturb PrPc function at the PC-12 cell surface, we pre-incubated NGF-primed (non-differentiated) or LN-differentiated cells with dye-labeled anti-PrPc antibodies and submitted cells to laser irradiation. The specific laser inactivation of PrPc at the surface of PC-12 cells promoted a significant inhibition of neurite extension (Fig. 3). Full inhibition of neurite outgrowth was probably not achieved because integrins have also been shown to mediate LN binding in this model [23]. Laser irradiation without antibodies, with MG-labeled anti-GST IgG (data not shown) or rabbit non-immune IgG (used as control), does not have effect on the neuritogenesis process. Furthermore, other neural adhesion molecules as L1 and NCAM-180 seem also to be closely related with neurite outgrowth control [17]. We have previously observed using flow cytometry [9] that NGF-treated PC-12 cells express 25% more PrPc than non-differentiated ones. Accordingly, NGF treatment also increases PrPc levels in the brains of neonatal hamsters [27] and in SV40 T-antigen immortalized mouse neuronal cell lines [28]. Thus, it is conceivable that the NGF-induced increase in cell surface PrPc (achieved by priming PC-12 cells with this soluble factor for 5 days) may facilitate differentiation after plating the cells on LN substrate.

To address whether PrPc function could modulate LN-in-

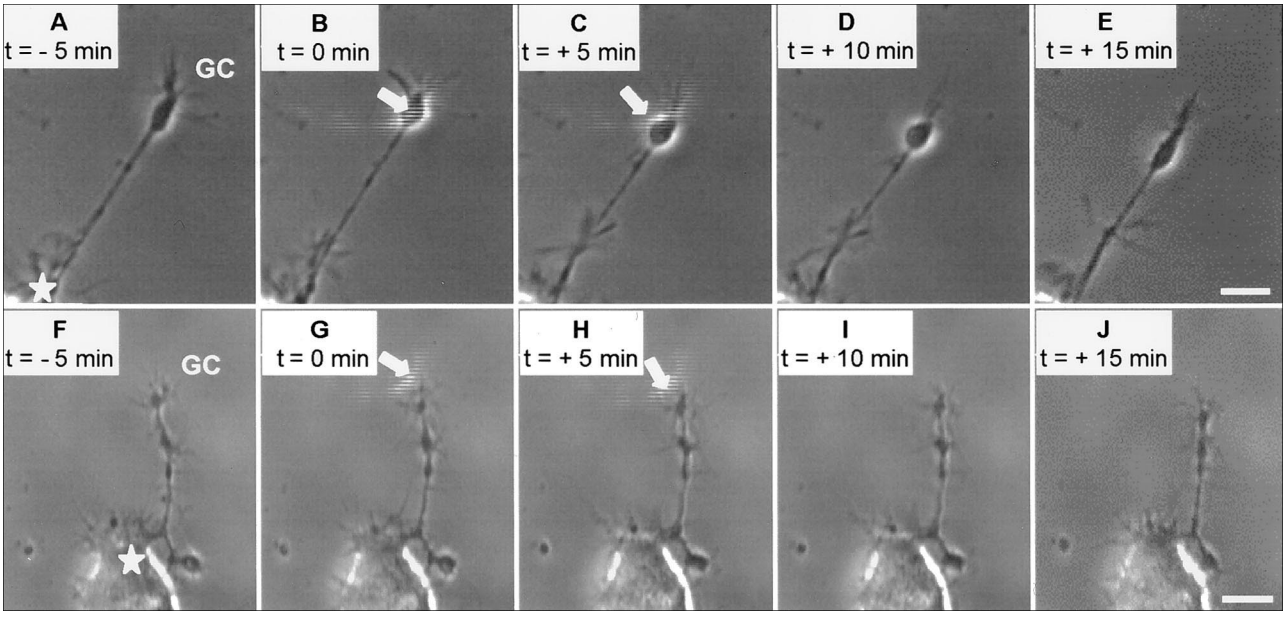

Fig. 4. Micro-scale CALI of PrPc causes LN-induced neurite retraction (A–E). A: the PC-12 cell incubated with chromophore-labeled IgG purified from anti-PrPc serum had the neurite first observed for 5 min; B and C: laser irradiation of the growth cone (GC), note that the neurite starts retracting at the end of this period; D and E: the neurite continues retracting in the 15 min period after laser irradiation. Micro-scale CALI with chromophore-labeled IgG purified from non-immune serum does not perturb neurite behavior (F–J). F: The PC-12 cell neurite is observed for 5 min; G and H: laser-irradiated for 5 min; I and J: neurite behavior was observed in the next 15 min after laser treatment. Neurite length measurements of nine anti-PrPc-treated and nine control cells using time-lapse video microscopy (one picture each 15 s, 80 pictures per studied cell) showed that those cells with inactivated PrPc at the growth cone suffered an average retraction of 1.86 ± 2.6 μm ($P = 0.017$, two-tailed Student t test), while control cells did not change their lengths (0.22 ± 0.66 μm). Arrows, period of time in which growth cones were maintained under photochemical inactivation of PrPc; * cell body; scale bar, 10 μm.

duced mature neurite behavior, we used the micro-scale CALI technique. As shown in Fig. 4A–E, focal laser inactivation of PrPc on the growth cone of pre-extended LN-induced neurites promotes a reduction of neurite length. Such a retraction was not achieved when micro-scale CALI was performed in the absence of antibodies, using dye-labeled anti-GST IgG (data not shown) or non-immune rabbit IgG (Fig. 4F–J). Indeed, the average of changes in the lengths of laser-inactivated PrPc neurites (-1.86 ± 2.6 μm) was significantly higher ($P = 0.017$, two-tailed Student t test) than the changes observed in control neurites (-0.22 ± 0.66 μm). Taken together, these results suggest that, besides LN–PrPc binding participation in the early phase of neurite outgrowth, maintenance of pre-extended PC-12 neurites is, at least in part, also modulated by LN–PrPc interaction.

Our results contribute to a better understanding of the participation of PrPc in neuronal survival by mediating adhesion and phenotypic modifications of such cells through its interaction with LN. However, further work will be necessary to identify the cell-signaling pathway triggered by LN–PrPc binding at the cell surface. This new emphasis on the role of PrPc may also help to clarify the pathogenesis of prion-related disorders.

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