neuroReport

Website publication 17 February 1998

NeuroReport 9, 709–712 (1998)

Functional thrombin receptor PAR1 in primary cultures of human glioblastoma cells

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IN this study we investigated primary cultures obtained from two glioblastomas surgically removed from a 64-year-old man and a 50-year-old woman, respectively. The presence of the tethered ligand thrombin receptor PAR1 (protease-activated receptor 1) in these cells was demonstrated at the level of receptor binding by using immunofluorescence studies with the monoclonal anti-PAR1 antibody Mab 31-2. Stimulation of human glioblastoma cells both wit hα-thrombin and the thrombin receptor activating peptide TRAP-6 resulted in a series of $[Ca^+]_i$ spikes as shown by confocal laser fluorescence microscopy with fluo-3 as calcium sensitive fluorescence indicator. This effect was completely blocked with the thrombin receptor antagonist peptide T1. Our results demonstrate functional thrombin receptors (PAR1) in primary cultures of human glioblastomas for the first time.

Key word s Calcium signaling; Glioblastoma α-Thrombin; TRAP-6

Introduction

It is well known that thrombin promotes tumor cell growth and adhesion as well as enhancing the metastatic capacity of tumor cells.[1–3] Since the effect of thrombin on the adhesion of rat carcinosarcoma and mouse melanoma cells to fibronectin has been shown to be mediated by the tethered ligand thrombin receptor PAR1 (protease-activated receptor 1),[4] its biological function in tumor cells has been suggested. mRNA encoding for prothrombin[5] and PAR1[6] is present in various regions in the central nervous system (CNS) and since astroglial cells in culture process and secrete thrombin,[7] suggesting a role for thrombin in brain development and plasticity. However, nothing is known about thrombin and thrombin receptors in brain tumors. As a consequence, we attempted to characterize PAR1 in primary cultures of human glioblastomas by immunofluorescence studies with a monoclonal anti-PAR1 antibody and measurement of free intracellular calcium mobilization induced b yα-thrombin and the thrombin receptor activating hexapeptide (TRAP-6).

Materials and Methods

Human α-thrombin (3700 NIH Units/mg protein) was purchased from Sigma Chemicals Co., fluo-3-acetoxymethylester from Molecular Probes, thrombin receptor antagonis (MSRPACPNBKYQ peptide T1) from Neosystem and human monoclonal anti-fibroblast Mab AS02 and human monoclonal anti-GFAP Mab from Dako Diagnostica GmbH. The thrombin receptor activating peptide TRAP-6 (SFLLRN) and the Mab 31-2 control peptide (KYEPFWEDEE) were provided from Dr Peter Henklein, Institute of Biochemistry, Charité, Berlin. The monoclonal anti-thrombin receptor Mab 31-2 directed against the hirudin-like domain KYEPFWEFDEE of the thrombin receptor N-terminus was a gift from Dr Vanitha Ramakrishnan, COR Therapeutics, Inc., South San Francisco.

Preparation of tumor cell culture Tumor tissue for cell culture was taken from glioblastomas surgically removed from a 64-year-old man and a 50-year-old woman, respectively. After removal of blood and blood vessels, the tumor tissue was mechanically dissected using scalpels. Subsequent trypsination (0.2% trypsin in calcium- and magnesium-free phosphate buffered saline) was performed for 2 5 min at 37°C. Trypsin was removed by centrifugation and resuspension in DMEM supplemented with 10% fetal calf serum. Cells were routinely cultured at 3 °C in a humidified atmosphere of 5% CO_2. The culture medium was changed every 2–3 days.

Immunochemistry: Cells were fixed with 3% paraformaldehyde and permeabilized with ice-cold 80% methanol. After incubation with RPMI 1640 supplemented with 10% FCS for 1 h, the preparation was incubated for 1 h at room temperature with either anti-thrombin receptor Mab 31-2, anti-GFAP and human anti-fibroblast Mab AS 02, respectively, in PBS/0.1% BSA (1:100). The preparation was then washed three times with PBS and was next exposed to the secondary antibody (fluorescein isothiocyanate-conjugated anti-mouse IgG at 1:100 and 0.1% BSA/PBS, Santa Cruz, USA or Cy3-conjugated anti-mouse IgG at 1:100 and 0.1% BSA/PBS, Dianova). For confocal microscopy, the preparation was mounted with 50% glycerol under a glass coverslip. A LSM 410 inverted microscope (Carl Zeiss) with an argon ion and helium–neon laser was used. Results were reproduced by at least three independent experiments.

Calcium measurements: Mobilization of $[Ca^+]_i$ was measured with fluo-3, a fluorescence indicator of free Ca^+ as described elsewhere.[8] Cells were grown on glass coverslips precoated with poly-L-lysine and washed twice with a buffer (pH 7.4) containing 10 mM HEPES, 145 mM NaCl, 0.5 mM Na_2HPO_4, 6 mM glucose, 1 mM $MgSO_4$, 1.5 mM $CaCl_2$. Cells were incubated for 15 min at 37°C in the same buffer containing 2.0 μM fluo-3 acetoxymethylester. After fluo-3 loading the cells were washed twice and reincubated in HEPES buffer. For measurement of free calcium mobilization in individual cells an inverted confocal laser scanning microscope (LSM 410, Carl Zeiss) was used. Fluorescence images were collected by using the 488 nm argon ion laser line. The intracellular calcium concentration was calculated using the equation $[Ca^+]_i = K_d \times (F - F_{min})/(F_{max} - F)$. The Ca^+ affinity of fluo-3 (K_d) is 400 nM.[8] F_{max} was obtained by addition of 10 mM ionomycin (+6 mM $CaCl_2$), F_{min} by addition of 20 mM EGTA.

Results

Immunofluorescence studies: First, presence of PAR1 was investigated in primary cultures of human glioblastoma cells by using a monoclonal antibody specific for the amino-terminus of the thrombin receptor. Figure 1 shows immunofluorescence staining of PAR1 by Mab 31-2 binding in the human glioblastoma cells. Enhancement of immunoreactivity with no definite detection of the exact receptor localization could be demonstrated. When Mab 31-2 was preincubated with the control peptide KYEPFWEDEE at concentrations > 50 μM, inhibition of the antibody binding was observed in glioblastoma cells (data not shown). In addition, a monoclonal anti-human GFAP antibody was used to proof astrocytic features of tumor cells. Anti-GFAP immunoreactivity could be observed in cells of similar morphology as those seen in Fig. 1 (data not shown).

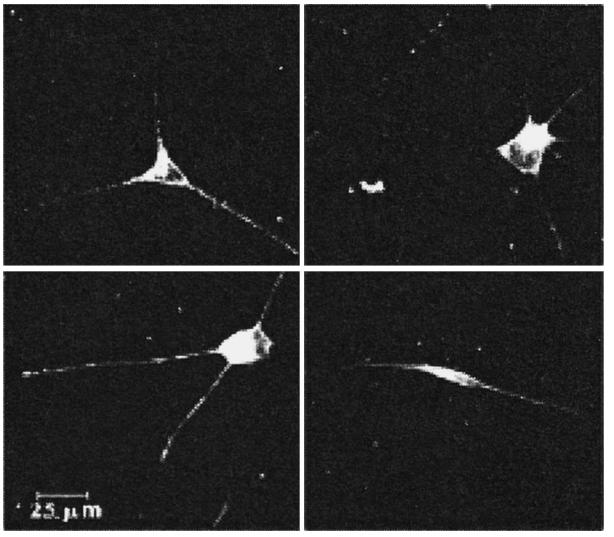

FIG. 1. Confocal microscopy of PAR1 in primary culture of human glioblastoma. Cells were treated with Mab 31-2 (1:100) and a secondary FITC-conjugated anti-mouse IgG. Fluorescence images were obtained with a confocal laser scanning microscope (LSM 410 invert). The picture is a representative of five independent experiments.

Intracellular calcium concentration: The effect of α-thrombin and TRAP-6 on intracellular Ca^+ mobilization in glioblastoma cells was investigated using confocal laser fluorescence microscopy in 25 individual cells. While four cells exibited no calcium signal after stimulation with α-thrombin or TRAP-6 (data not shown), calcium mobilization could be observed in 21 cells (Fig. 2). These glioblastoma cells did not respond to α-thrombin or TRAP-6 with a single monophasic Ca^+ increase, but produced a series of $[Ca^+]_i$ spikes (Fig. 3). This α-thrombin- and TRAP-6-induced $[Ca^+]_i$ mobilization could be inhibited with the thrombin receptor antagonist peptide T1 dose-dependently, as shown in Fig. 4 for TRAP-6-induced Ca^+ response. In further investigations, the cells used for calcium experiments were characterized by immunofluorescence staining using the monoclonal anti-fibroblast antibody Mab AS 02. No fibroblast immunoreactivity could be observed for the cells used in measurement of intracellular calcium mobilization (data not shown).

Discussion

First results on cellular functions of the tethered ligand thrombin receptor PAR1,[9] which was recently

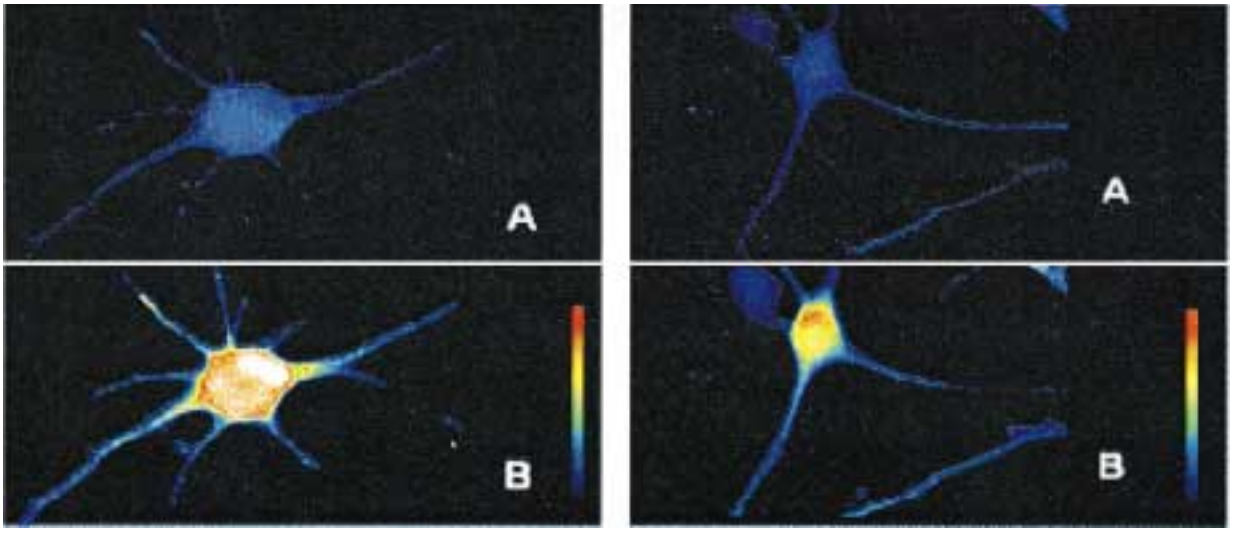

FIG. 2. TRAP-6- and α-thrombin-induced $[Ca^+]_i$ mobilization in individual human glioblastoma cells. Human glioblastoma cells were loaded with fluo-3 (2.0 μM) for 15 min at 37°C. Times after stimulation with TRAP-6 (final conc. 1.0 μM) or α-thrombin (final conc. 1.0 nM) are indicated. Blue represents low and red high fluorescence. (**A**) Glioblastoma cell before stimulation; (**B**) glioblastoma cell 30 s after application of TRAP-6 (left) or α-thrombin (right).

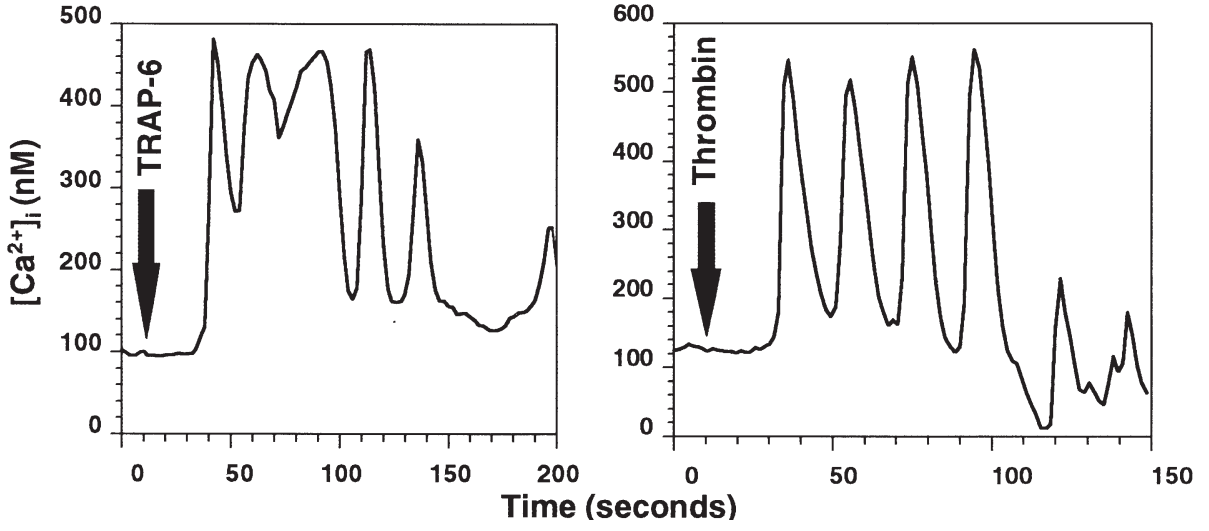

FIG. 3. $[Ca^+]_i$ oscillations in individual TRAP-6- or α-thrombin-stimulated human glioblastoma cells. These data are taken from the same cells shown in Fig. 2 and demonstrate the time course of changes in $[Ca^+]_i$ calculated from the digital images of fluo-3 fluorescence. The $[Ca^+]_i$ levels were calibrated using the equation $[Ca^+]_i = 400\ nM \times (F - F_{min})/(F_{max} - F)$.

cloned and charcterized in cancer cell lines,[4] suggest its involvement in cancer processes. We characterized PAR1 in primary cultures derived from human glioblastoma by antibody binding using a monoclonal antibody specific for the amino-terminus (hirudin-like domain) of the thrombin receptor.[10] Mab 31-2 binds the human thrombin receptor PAR1 with high affinity (about 1 nM).[11] Although the thrombin receptor PAR1 is known to be a cell surface receptor, recent results suggest the existence of intracellular thrombin receptor pools in endothelial HUVEC cells.[12] In human glioblastoma cells, the exact localization of thrombin receptors remains unknown and has to be elucidated in further investigations especially using electron microscopy. The finding that the Mab 31-2 control peptide KYEPFWEDEE inhibited Mab 31-2-induced immunofluorescence is a further evidence for the presence of PAR1 in human glioblastoma cells.

To demonstrate that these binding sites constitute functional thrombin receptors their connection with the calcium system was investigated. Stimulation of individual human glioblastoma cells both with α-thrombin or TRAP-6 resulted in mobilization of free intracellular calcium with a series of $[Ca^+]_i$ spikes. In former studies, a number of mechanisms have been

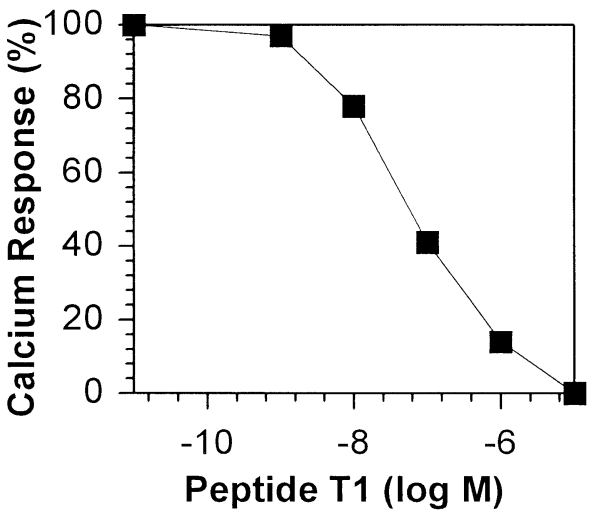

FIG. 4. Dose–response curve of the thrombin receptor antagonist peptide T1 in inhibiting TRAP-6-induced $[Ca^+]_i$ mobilization in glioblastoma cells. Cells were preincubated with peptide T1 for 30 min. Calcium measurements were performed as described in Materials and Methods.

proposed to account for $[Ca^+]_i$ oscillations induced by agonists which act through inositol (1,4,5)triphosphate ($InsP_3$), including feed back inhibition of $InsP_3$ formation by protein kinase C, feed forward activation of phospholipase C by Ca^+, inhibition of $InsP_3$ action by Ca^+, and Ca^+-induced Ca^+ release (CICR).[13–15] The mechanisms involved in PAR1-mediated calcium waves in human glioblastoma are still undefined and have to be investigated.

Since TRAP-6 mimicked the effect of α-thrombin on $[Ca^+]_i$, it may be concluded that PAR1 is involved in thrombin-induced calcium signaling in human glioblastoma cells. This was supported by the finding that the thrombin receptor antagonist peptide T1 blocked the α-thrombin- and TRAP-6-induced effects on intracellular calcium in human glioblastoma cells. The true neuroepithelial nature of the cells was confirmed by immunocytochemistry, as these were anti-fibroblast antibody-negative and GFAP-positive.

Conclusion

Our results indicate for the first time that primary cultured human glioblastoma cells express the thrombin receptor PAR1. They also indicate that the receptors are functional in mediating calcium signaling in individual human glioblastoma cells. A role of the thrombin receptor PAR1 in glial brain tumors is therefore hypothesized. Further experiments are necessary to elucidate the significance of functional PAR1 in glioblastoma.

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ACKNOWLEDGMENTS: We are grateful to Dr Peter Henklein, Institute of Biochemistry, Medical Faculty (Charité) of the Humboldt University of Berlin for providing TRAP-6 and KYEPFWEDEE and Dr Vanitha Ramakrishnan, COR Therapeutics Inc., South San Francisco for the gift of Mab 31-2. We thank Mrs Raabe and Mrs Faeseke for excellant technical assistance. This work was supported by the Deutsche Krebshilfe (10-1254 Ka 2).

Received 12 November 1997;
accepted 16 December 1997