Exogenous Platelet-derived Growth Factor (PDGF) Induces Human Astrocytoma Cell Line Proliferation

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Abstract. *Platelet-derived growth factor receptors (PDGFR) regulate several processes in normal cells including cellular proliferation, differentiation and migration, and are widely expressed in a variety of malignancies. In astrocytoma, PDGF ligand and receptor are often overexpressed and PDGFR activity deregulation has been linked to pathogenesis. The issue of the functional capacity of PDGFR has only occasionally been addressed in glioma cells by measuring the proliferative response induced by exogenous PDGF. In the present study, PDGFRα expression was evaluated in human grade 2 and 4 astrocytoma cell lines and tissue specimens by immunocytochemistry. The receptor responsiveness to exogenous PDGF was determined in astrocytoma cells with an MTT assay. It was found that astrocytoma cells express PDGFRα and respond to PDGF mitogenic action in a grade-dependent manner. The receptor was found to be functional since it induced cell proliferation at different ligand concentrations. We can thus conclude that the proliferative response of human astrocytoma cells is related to their malignancy and receptor status before PDGF stimulation, suggesting a role for PDGFRα inhibitors as blockers of malignant cell proliferation.*

Tyrosine kinase proteins constitute a large family of molecules that behave as important regulators of intracellular signal transduction pathways (1). Their activities regulate a range of fundamental cellular functions such as proliferation, differentiation, apoptosis, adhesion and migration (2, 3). The deregulation of protein kinase activity has been shown to play a central role in the pathogenesis of many human cancers (4-6).

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Platelet-derived growth factor (PDGF), a member of this family, is a potent mitogen for mesenchymal cells, playing an essential role in cellular growth, proliferation, differentiation and migration (7). PDGF is produced by a number of cell types, including platelets, endothelial cells, smooth muscle cells, macrophages and glial cells (8), and its function is linked to interaction with specific cell surface receptors. Active PDGF molecules show a dimeric structure that includes A, B, C and D chain homodimers, and AB heterodimer. These isoforms bind two structurally and functionally similar PDGF receptors, denoted α and β, with different specificity (9). The α-receptor binds three different isoforms (PDGF-AA, -AB, -BB) with high and comparable affinity, in the same way as the β-receptor binds PDGF-BB (10). PDGF binding to its receptor causes receptor dimerization, leading to autophosphorylation and activation of a number of intracellular signalling pathways that induce cell proliferation and several other crucial processes (11, 12). In astrocytoma, several alterations of the PDGF/PDGFR signal transduction pathway, including protein overexpression, and autocrine and paracrine ligand stimulation, have been described (13). In fact, different studies have demostrated that normal astrocytes in culture express functional PDGFRs and are targets for PDGF action; moreover the overexpression of the PDGF gene and the coexpression of PDGFRs represent an important step in malignant astrocytoma development and progression (13, 14).

Astrocytomas are aggressive brain tumours that numerically exceed all other primary intracerebral neoplasms, characterized by infiltration of surrounding brain tissue, fast tumour growth and fatal outcome within months or a few years (15). According to WHO, astrocytomas are classified into pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma multiforme (GBM) (grade IV) (16).

A few previous studies assessed the functional capacity of PDGFR on human glioma by measuring cellular proliferative response to exogenous PDGF (9, 17). The aims

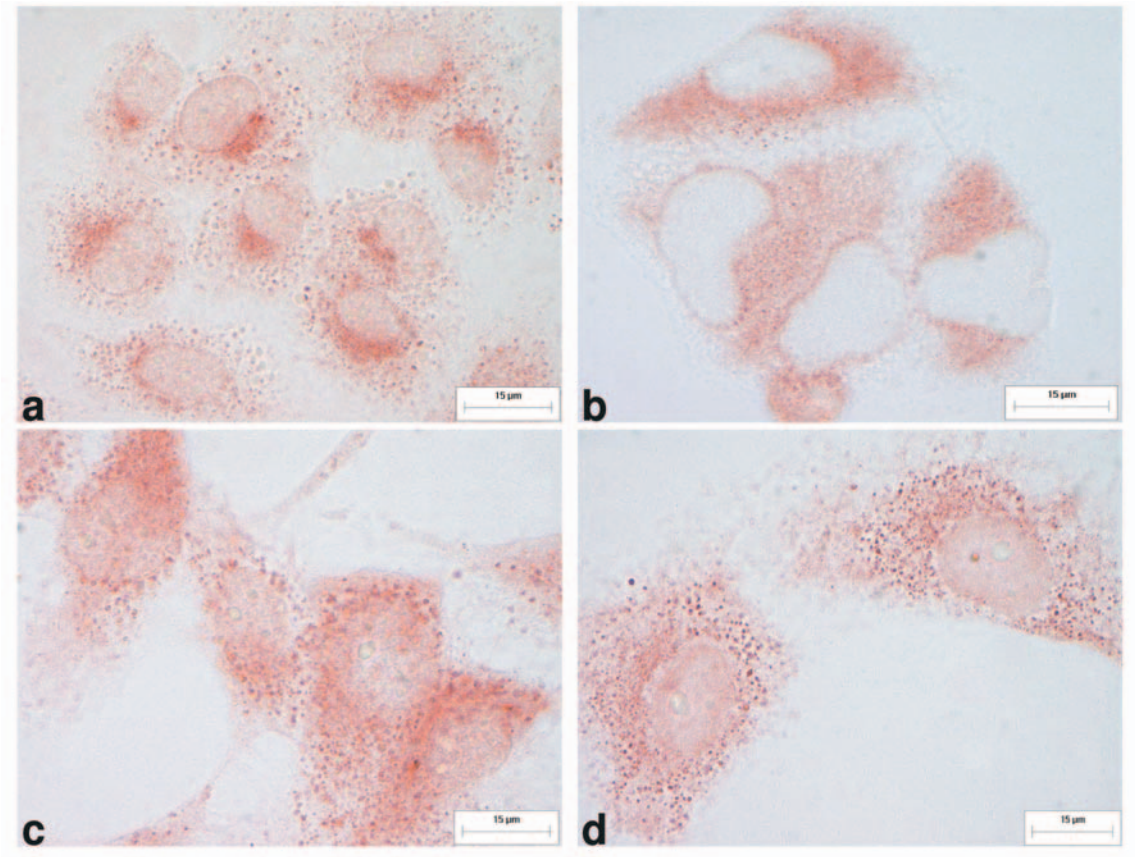

Figure 1. *PDGFRα expression in astrocytoma cells. Representative immunocytochemical stainings of PDGFRα in human astrocytoma cell lines (original magnification x1000): (a) IPDCC-A2; (b) T98G; (c) A172; (d) PRT-Hu2.*

of the present study were to evaluate PDGFRα immunocytochemical expression in astrocytoma biopsies and cell lines, to investigate the cell line response to exogenous PDGF and to analyse the relationship between PDGFRα expression and its functional capacity.

Materials and Methods

Cell cultures. The human low-grade astrocytoma cell line IPDDC-A2 and glioblastoma cell lines A172 and T98G were purchased from the European Collection of Cell Culture (ECACC, UK). The glioblastoma cell line PRT-Hu2 was obtained from a patient biopsy (18).

A172 and IPDDC-A2 cells were routinely cultured as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/l L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). T98G and PRT-Hu2 were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 2 mmol/l L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich). Cells were maintained in a humidified 5% CO_2 atmosphere at 37˚C and the medium was replaced every 2 to 3 days.

Tissue specimens. Archival formalin-fixed, paraffin-embedded human astrocytoma sections were obtained from diagnostic biopsies or resection specimens from 10 patients treated at the Foundation IRCCS Policlinico S. Matteo (Pavia, Italy), after approval of the local ethics committee. All cases included in this study were histologically confirmed by a neuropathologist and classified according to WHO classification as diffuse astrocytoma (DA, n=5) or glioblastoma multiforme (GBM, n=5).

Immunocytochemistry. Immunocytochemical analysis with prediluted monoclonal anti-human PDGFRα antibody (GeneTex, S. Antonio, TX, USA) was performed on astrocytoma cell lines and sections.

Cells ($3x10^4$) were seeded in duplicate on cover slips and incubated with complete fresh medium for 24 h. Thereafter, cells were fixed in cold 70% ethanol for 10 min at room temperature. The cells were incubated with the primary antibody for 1 h at room temperature. Immunostaining was performed with EnVision™ (DakoCytomation, Carpinteria, CA, USA) according to the manufacturer's protocol.

In addition, the immunocytochemical study was performed on diffuse astrocytoma (DA) samples: 5-μm paraffin-embedded sections were dewaxed in xylene and rehydrated through a series of ethanols to water. Antigen retrieval was obtained in 10 mM citrate

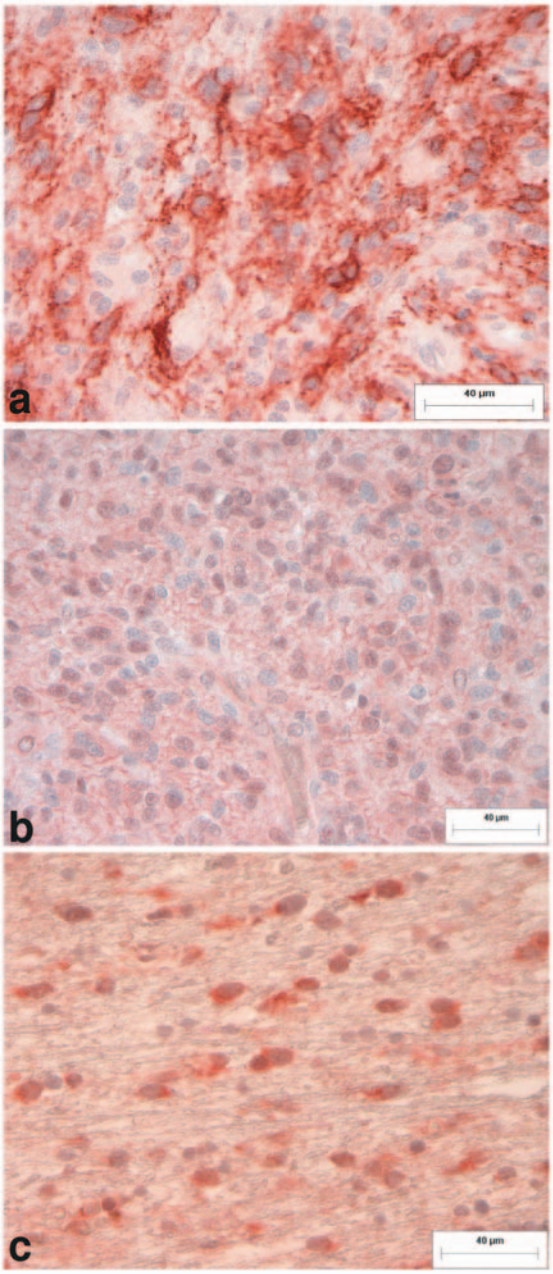

Figure 2. *PDGFRα expression in astrocytoma sections. Immunocytochemistry for PDGFRα in glioblastoma multiforme (a, b) and diffuse astrocytoma (c) formalin-fixed, paraffin-embedded sections (original magnification x400): cytoplasmatic and membrane staining with high (a) and moderate (c) reaction intensity and solely membrane pattern staining (b).*

buffer (pH 6.0) for 15 min (3x5 min) in a microwave at 750 W. After cooling to room temperature for 20 min, sections were rinsed with phosphate-buffered saline (PBS). Subsequently, the sections were incubated with anti-PDGFRα antibody for 1 h at room temperature. Detection of immunostaining was performed with EnVision™ (DakoCytomation) according to the manufacturer's protocol, and counterstaining with Mayer's hematoxylin. Negative controls were obtained by omitting the primary antibodies.

In astrocytoma samples, the immunocytochemical staining was independently assessed by two observers at 400-fold magnification, using a semi-quantitative 4-score intensity scale (0, negative; 1, light; 2, moderate; 3, strong; 4, very strong). Interobserver variation of less than 5% was seen.

MTT assay. The effects of PDGF on astrocytoma cell proliferation were determined in 96-well plates by MTT assay. A total of 10^4 cells in complete fresh medium were distributed into each well of a flat-bottomed microplate and incubated overnight. The medium was removed and cells incubated for 24 and 48 h with serum-free medium containing 10 or 20 or 30 ng/ml PDGF (Sigma-Aldrich). Control cells were maintained in serum-free medium. Thereafter the medium was removed and MTT solution (0.5 mg/ml in RPMI without phenol red) was added to each well and incubated for 1 h. The MTT solution was then removed and the converted dye was solubilized by addition of acidic isopropanol (0.04 N HCl in absolute isopropanol). The absorbance (optical density, OD) of converted dye was measured at 540 nm with a microplate reader. Each experiment was perfomed in duplicate.

Statistical analysis. The statistical significance of experimental results was assessed with the unpaired Student's *t*-test. One-way ANOVA was performed to determine whether the proliferation differences among cell lines, under increasing PDGF concentrations, were statistically significant. A p value of <0.05 was considered significant.

Results

PDGFRα immunocytochemical expression. We firstly investigated whether astrocytoma cells express PDGFRα. As shown in Figure 1, PDGFRα immunocytochemical reactions showed a well defined reaction pattern, with very low background stain, with some difference related to malignancy grade. In the low-grade astrocytoma cell line IPPDC-A2, PDGFRα expression was of moderate intensity and localized to the perinuclear cytoplasm (Figure 1a). In T98G, A172 and PRT-Hu2 cell lines (GBM), diffuse cytoplasmic reactivity was observed, with slight membrane enhancement in some cases (Figure 1b-d).

Subsequently, we evaluated PDGFRα expression in astrocytoma samples. Immunohistochemistry in DA and GBM samples revealed a PDGFRα expression score ranging from 1 to 3 and 2 to 4, respectively (Table I). All samples were immunoreactive for PDGFRα. Four GBM samples showed cytoplasmatic and membrane staining (Figure 2a) with different intensities among sections, while one showed exclusive membrane expression (Figure 2b). The PDGFRα reaction pattern seen in 5 DAs was similar to that observed in GBM samples, although with a lower intensity (Figure 2c). Moreover, in DA we observed remarkable staining heterogeneity within the same section.

Table I. *PDGFRα immunohistochemical scores. Immunohistochemical intensity score of PDGFRα in astrocytoma formalin-fixed, paraffin-embedded sections.*

Samples	Score
DA	
1	2
2	1
3	2
4	1
5	3
GBM	
1	3
2	2
3	3
4	4
5	4

DA: diffuse astrocytoma; GBM: glioblastoma multiforme; 0 = negative; 1= light; 2 = moderate; 3 = strong; 4 = very strong.

Effects of PDGF on proliferation. The effect of PDGF on cellular proliferation was assessed by the MTT assay. As shown in Figure 3, PDGF stimulated proliferation in all cell lines in a concentration-dependent manner. At the lowest PDGF concentration, only A172 cells significantly increased their proliferation rate as compared to under the serum-free condition, both after 24 and 48 h of incubation; 20 ng/ml of PDGF caused a significant increase of cell proliferation in IPDDC-A2 cells at 24 and 48 h and in PRT-Hu2 at 48 h. At 30 ng/ml we observed a significant ($p<0.05$) increase in cell proliferation in all cell lines at both time points.

In IPPDC-A2 and PRT-Hu2 cell lines, the effects of PDGF on cellular proliferation were found to be time-dependent (Figure 3a, d). In fact, a significant difference in cell proliferation between 24 and 48 h was observed in IPDDC-A2 cells at 20 and 30 ng/ml, and at all tested concentrations in PRT-Hu2 cells. In the other two cell lines, no significant differences were observed between 24 and 48 h at any concentration.

In addition, at 24 h we saw a significant difference ($p<0.05$) among glioblastoma and low grade astrocytoma cell line responses at all PDGF concentrations, but this significance was lost at 48 h for 20 and 30 ng/ml (Figure 4).

Discussion

PDGFR is a growth factor receptor with intrinsic tyrosine kinase activity that is deregulated in several human diseases, including tumours. In gliomas, an increased expression of PDGF and its receptor has been described, suggesting that autocrine and paracrine mechanisms of activation of this signalling pathway might play a role in glioma cell proliferation (19).

In this study, we have described immunocytochemical PDGFRα expression in astrocytoma cell lines and tumor tissue. Several studies have already reported the presence of this receptor in human astrocytoma (9, 20, 21). In the present study, we focused our attention on its immunocytochemical expression in relation to its functional capacity in four human astrocytoma cell lines (IPDDC-A2, A172, T98G and PRT-Hu2), measuring the proliferative response induced by exogenous PDGF with the MTT test.

Firstly, we analyzed PDGFRα expression in astrocytoma samples to confirm its *ex vivo* presence. In accordance with other studies, we observed that GBM (IV grade) immunohistochemical staining intensity was higher than that found in DA (grade II) samples (22). The analysis of PDGFRα expression in astrocytoma cell lines showed a grade-dependent staining intensity and localization. Interestingly, we observed some correlation between the proliferative response of astrocytoma cells to exogenous PDGF stimulation and PDGFRα immunocytochemical localization. In IPDDC-A2 cells, immunocytochemistry showed that the receptor protein was mainly present in the cytoplasm around the nucleus, where it was likely associated with the endosomal compartment (Figure 1a). This suggests that the cells are in a receptor down-regulation status. In fact, under physiological conditions, endocytosis negatively regulates tyrosine kinase signalling pathways: ligand-induced down-regulation leads to a significant reduction of both the total receptor pool (23) and growth factor stimulation. In particular, the binding of PDGF on its surface receptors induces rapid endocytosis of ligand-receptor complexes *via* clathrin-coated pits. Internalized receptors pass through the endosomal compartment where they are efficiently sorted to the lysosomal degradation pathways. The pattern of PDGFRα expression observed in IPDDC-A2 cells suggests that these cells may be able to balance endogenously produced PDGF signalling, inducing a receptor down-regulation, analogously to what has been described in other glioma cell lines (9). In IPDDC-A2 cells, PDGFRα expression is moderate if compared to glioblastoma lines, therefore these cells seem to be able to balance PDGF signalling, inducing receptor down-regulation. In contrast, glioblastoma cells are not in a down-regulated condition, as highlighted by our immunocytochemical study, likely because PDGFR overexpression causes an inefficient receptor down-regulation due to the inability of the endocytic machinery to work with large amounts of activated receptors (24).

Therefore, the higher proliferative response of glioblastoma cells to PDGF stimulation as compared to that of the low grade astrocytoma cell line, might be explained by the larger receptor pool present on the cell membrane surface, as documented both in immunohistochemical and immunocytochemical reactions. In fact, in IPDDC-A2 the

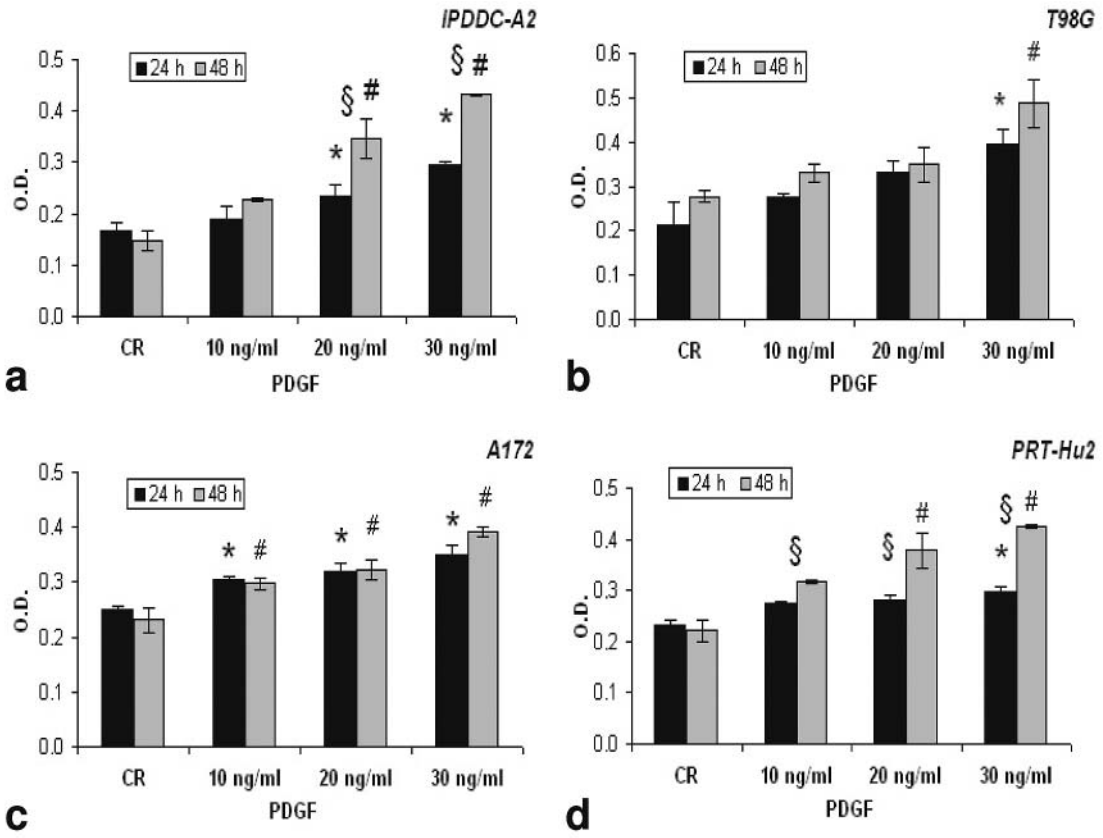

Figure 3. *Effect on proliferation of human astrocytoma cell lines under control conditions (CR) or in the presence of PDGF (10, 20 and 30 ng/ml): (a) IPDCC-A2; (b) T98G; (c) A172; (d) PRT-Hu2. Cell proliferation was evaluated measuring the absorbance (optical density, O.D.) at 540 nm. (* $p<0.05$ treated vs. control at 24 h; # $p<0.05$ treated vs. control at 48 h; § $p<0.05$ 48 h vs. 24 h).*

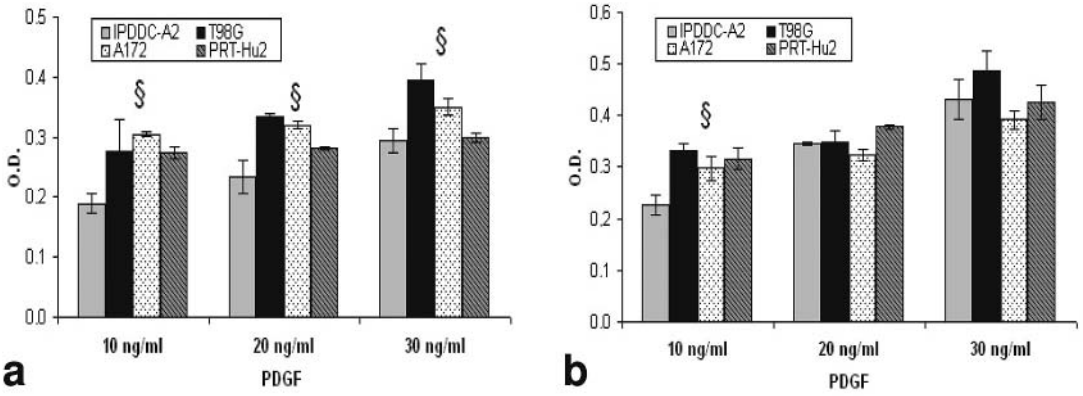

Figure 4. *Comparison of the effect on proliferation* of *different PDGF concentrations (10, 20 and 30 ng/ml) of four human astrocytoma cell lines (IPDCC-A2, T98G, A172, PRT-Hu2) (a) 24 h and (b) 48 h after stimulation. Cell proliferation was evaluated measuring the absorbance (optical density, O.D.) at 540 nm. (§ $p<0.05$; ANOVA).*

receptor down-regulation might reflect a reduced number of functional receptors on the membrane and consequently a quantitatively lower response to exogenous PDGF stimulation. The observation that 48 h after PDGF stimulation IPDDC-A2 cells responded similarly to glioblastoma cells led us to hyphothesize that after the down-regulation, newly synthesized PDGF receptors on the membrane surface are displayed.

Concerning PRT-Hu2 cells, we observed a time-dependent response with PDGF without a down-regulation of the receptor. This effect is likely to be due to the heterogeneity of this cell line as shown by flow cytometry (data not shown); therefore we hypothesize that the overall PDGF effect is the result of an average of the effects induced in each cell population related to their PDGF sensitivity.

The results of the present study indicate that astrocytoma cell lines express PDGFRα and respond to PDGF mitogenic action. Therefore these receptors are functional since they mediate a proliferative response with increasing PDGF concentrations. These observations imply that PDGFRα inhibitors might be candidates as effective blockers of malignant cell proliferation. In fact, there is increasing evidence supporting a role for PDGFR antagonists in the treatment of cancer patients. In astrocytoma cells, where the PDGF/PDGFR signal trasduction pathway is important, PDGFR blockage can provide a new therapeutic approach.

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