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A glioma-derived analog to platelet-derived growth factor: Demonstration of receptor competing activity and immunological crossreactivity

(cell culture/^{125}I-labeled platelet-derived growth factor binding/radioimmunoassay/immunoprecipitation/NaDodSO$_4$/gel electrophoresis)

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ABSTRACT **A human clonal glioma cell line, U-343 MGa Cl 2, cultured under serum-free conditions, was found to release a factor that competed with ^{125}I-labeled platelet-derived growth factor (^{125}I-PDGF) for binding to human foreskin fibroblasts. The concentration of competing activity in conditioned medium was equal to 20–30 ng of PDGF per ml. The PDGF receptor competing activity had an elution position on Sephadex G-200 close to that of tracer PDGF. The same fractions in the chromatogram also contained growth-promoting activity and material active in a PDGF radioimmunoassay. Incubation of partially purified, ^{125}I-labeled glioma factor with fibroblasts, or a rabbit anti-PDGF serum, led to the selective binding of a component with an estimated M_r of 31,000, as shown by NaDodSO$_4$/gel electrophoresis under nonreducing conditions. After reduction this component migrated as a M_r 18,000 protein. Thus, the behavior in NaDodSO$_4$/gel electrophoresis was similar to that of PDGF. Furthermore, incubation of partially purified glioma factor with immobilized PDGF antibodies markedly decreased the amount of PDGF receptor competing activity remaining in the supernatant. These results suggest that the factor produced by glioma cells has structural, immunological, and functional resemblance to PDGF. We previously reported that a human osteosarcoma cell line produces a PDGF-like molecule with growth-promoting activity. Taken together with the recent finding that PDGF is homologous to the transforming gene product of simian sarcoma virus, our present data give additional support for the idea that an autocrine activation of the PDGF receptor may be operational in the growth of human tumors of mesenchymal or glial origin.**

Platelet-derived growth factor (PDGF), a cationic protein of $M_r \approx 30{,}000$, is a potent growth factor for connective tissue-derived cells and glial cells in culture (1–3). *In vivo*, PDGF is probably synthesized in the megakaryocyte, stored in the platelet α-granules, and released by the platelet-release reaction (4–6). Consequently, under normal conditions cells may not be exposed to PDGF; only when platelets degranulate does PDGF become free to diffuse into tissue. The storage form of PDGF in conjunction with its restricted cell specificity implies a physiological role of PDGF in the stimulation of cell regeneration in response to tissue damage.

A partial amino acid sequence of human PDGF has recently been determined (7–9). The obtained sequence shows virtual identity with that of p28^{sis}, the putative transforming protein of simian sarcoma virus (10). This finding suggests that neoplastic transformation involves the expression of growth factors, which may be encoded by oncogenes. Recent studies have clearly shown that certain tumor cells do release growth factors. One example is the finding of transforming growth factors (TGFs) produced by virus-transformed cells (11–13). TGF not only stimulates cell proliferation but also, in certain systems, is capable of inducing anchorage-independent growth. Because TGF blocks the binding of epidermal growth factor (EGF) (11), some of its cellular effects are probably mediated by the EGF receptor; however, recent data derived from cross-linking studies indicate that TGF also binds to a cell receptor, distinct from the EGF receptor (14). Furthermore, growth factors related to PDGF have been isolated from human osteosarcoma cells (15) and from simian virus 40 (SV40)-transformed baby hamster kidney (BHK) cells (16, 17).

Recent improvements in PDGF radioligand techniques (18–22), have enabled us to investigate whether PDGF or PDGF-like factors are produced by human tumor cells. We now report on a human clonal glioma cell line (U-343 MGa Cl 2) that releases a M_r 31,000 growth factor with structural and immunological characteristics in common with PDGF.§

MATERIALS AND METHODS

Cell Culture. The *in vitro* characteristics of the human clonal glioma cell line U-343 MGa Cl 2 have been described (24). Human foreskin fibroblasts (line AG 1523) were purchased from the Human Mutant Cell Repository, Institute for Medical Research (Camden, NJ). Cells were routinely grown in 10-cm Petri dishes (Nunc) in Eagle's minimum essential medium, supplemented with 10% newborn calf serum (GIBCO) and antibiotics (100 units of penicillin and 50 μg of streptomycin per ml). Cultures were maintained at 37°C in humidified air containing 5% CO_2. Cultures were subcultivated once or twice a week at 1:2 split ratio using 0.2 mg of EDTA per ml and 2.5 mg of trypsin (Difco) per ml in phosphate-buffered saline as detaching agents.

For the harvest of larger amounts of serum-free conditioned media from U-343 MGa Cl 2 cultures, cells were transferred to Falcon plastic roller bottles (850 cm^2) at 1:5 split ratio (cm^2/cm^2) using 100 ml of Eagle's medium, supplemented with 10% newborn calf serum, antibiotics, and 10 mM Hepes (pH 7.4) per bottle. Roller bottles were incubated at 37°C and rotated at 0.5 rpm in a Bellco Technorama apparatus. Medium was changed twice a week. When the cells had grown to confluence, the rollers were washed three times with phosphate-buffered saline and incubated with 100 ml of serum-free F-10 medium per bottle. Medium was harvested twice a week as long as the cells appeared viable (1–2 months). Detached cells and debris were removed by centrifugation (10,000 × *g* for 10 min) and conditioned media were stored in plastic bottles at −20°C until processed further. For

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Abbreviations: PDGF, platelet-derived growth factor; ^{125}I-PDGF, ^{125}I-labeled PDGF; TGF, transforming growth factor; EGF, epidermal growth factor.

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§A preliminary report of this work has been presented (23).

small-scale experiments, serum-free media were harvested in an analogous way from cells grown in 10-cm Petri dishes.

PDGF. Pure PDGF was prepared as described (25) and radiolabeled using the chloramine-T method to a specific activity of ≈20,000 cpm/ng (18).

Assay for PDGF Receptor Competing Activity. PDGF receptor competing activity was measured on human foreskin fibroblasts. Cells were seeded in 12-well plates, grown to confluence, and washed once with binding buffer (phosphate-buffered saline containing 1 mg of human serum albumin per ml/0.01 mg of $CaCl_2 \cdot 2H_2O$ per ml/0.01 mg of $MgSO_4 \cdot 7H_2O$ per ml). Test samples (4°C), diluted in binding buffer, were then added and the plates were incubated at 4°C for 2 hr. Cells were washed 3 times with binding buffer. Each well then received 0.5 ml of binding buffer containing 5 ng (100,000 cpm) of ^{125}I-labeled PDGF (^{125}I-PDGF). After incubation for 1 hr at 4°C, cultures were washed 6 times with binding buffer containing 1% newborn calf serum instead of albumin. Cell-associated radioactivity was extracted with 0.5 ml of lysis buffer (1% Triton X-100/20 mM Hepes, pH 7.4/10% (vol/vol) glycerol, supplemented with 0.1 mg of bovine serum albumin per ml). The Triton lysate was sampled after 20 min and the radioactivity was determined in a gamma spectrometer. Nonspecific binding was determined as the amount of ^{125}I-PDGF bound in the presence of a 50-fold molar excess of unlabeled partially purified PDGF. PDGF receptor competing activity of samples was converted to equivalent ng/ml of PDGF, using a standard curve constructed from results obtained with pure unlabeled PDGF (5–100 ng per ml).

PDGF Radioimmunoassay. Antibodies against pure PDGF were raised in a rabbit as described (19). The immunoglobulin fraction, purified using *Staphylococcus* protein A Sepharose, was used to establish a radioimmunoassay against PDGF. Briefly, 96-well Multiwell Disposo-Trays (Linbro) were coated with anti-PDGF immunoglobulins (100 μl per well, 0.25 mg per ml in phosphate-buffered saline) for 30 min at room temperature. After washing 3 times in phosphate-buffered saline, test samples (100 μl), diluted in washing buffer (0.5 M NaCl/0.01 M phosphate buffer, pH 7.4/0.5% bovine serum albumin/0.1% Tween 80) were added. The plates were then incubated for 16 hr at 4°C under continuous shaking. At this time, test samples were removed and the wells received 100 μl of ^{125}I-PDGF (20,000 cpm) in washing buffer and incubation was prolonged for another 2 hr. After washing 3 times with washing buffer, immobilized radioactivity was released by incubation with 0.3 M NaOH (100 μl per well) for 10 min at room temperature. The assay was calibrated with pure PDGF (0.5–50 ng).

Assay for Growth-Promoting Activity. Growth-promoting activity was determined using a method of [^{3}H]thymidine incorporation in serum-free cultures of human foreskin fibroblasts (line AG 1523). Cells were plated in Eagle's minimal essential medium/10% newborn calf serum, at a split ratio of 1:10, in Nunc 35-mm dishes. After 4 days of incubation, medium was removed and the cultures were incubated for 2 days in serum-free MCDB 105 medium with a decreased concentration of Ca^{2+} (0.5 mM). Test samples were then added along with [^{3}H]thymidine (specific activity, 5 Ci/mmol, 1 Ci = 37 GBq; final concentration, 0.02 μCi/ml). After 2 days of incubation, cultures were extracted with 10% trichloroacetic acid, washed in running tap water, and lysed with 0.3 M NaOH/1% $NaDodSO_4$ in water. Radioactivity was determined in a liquid scintillation counter. The activity of test samples was compared to that obtained with various concentrations of pure PDGF (0.5–10 ng per ml).

Partial Purification of the PDGF Analog Released by a Human Glioma Cell Line. Six liters of serum-free conditioned medium from U-343 MGa Cl 2 cultures were harvested as described above. The active component was then concentrated by adsorption to Sulphadex beads (sulphated Sephadex G-50 medium; ref. 26); the conditioned medium was incubated with 120 ml of Sulphadex beads overnight at 4°C under continuous shaking. The gel was then collected by sedimentation and poured into a column. After washing with 50 ml of phosphate-buffered saline, the column was eluted with 300 ml of 2 M NaCl/0.01 M phosphate buffer, pH 7.4. Solid ammonium sulfate was then added to the eluate to 70% saturation, and the precipitate was collected by centrifugation at 10,000 × *g* for 30 min. It was then dissolved in 1.5 ml of 1 M NaCl/0.01 M phosphate buffer, pH 7.4, and applied to a Sephadex G-200 column (1 × 145 cm) equilibrated with the same buffer. The column was eluted at a flow rate of 4 ml per hr and effluent fractions were analyzed for protein, growth-promoting activity, and for radioreceptoractive and radioimmunoactive PDGF.

Cell Binding of Radiolabeled Partially Purified Material. Ten micrograms of protein derived from the peak fractions of the Sephadex G-200 chromatogram (75–87 ml eluted volume) was radiolabeled with ^{125}I according to the procedure used for the radiolabeling of PDGF (18). ^{125}I-labeled partially purified material (1.5×10^6 cpm) or ^{125}I-PDGF (10^5 cpm) was exposed to human foreskin fibroblasts (AG 1523) in 0.5 ml of binding medium for 2 hr at 4°C in the presence or absence of unlabeled PDGF (1 μg/ml). After washing 5 times with binding medium, cells were removed from the culture dishes with a rubber policeman and collected by centrifugation at 10,000 × *g* for 1 min. The cell pellets were then extracted with 80 μl of 1% Triton X-100/10% glycerol/20 mM Hepes, pH 7.4. After 15 min at 4°C, samples were centrifuged at 10,000 × *g* for 2 min, and the supernatant was incubated with 80 μl of 3.6% $NaDodSO_4$/80 mM Tris·HCl, pH 8.8/0.01% bromophenol blue for 3 min at 95°C. Samples were then divided into two equal parts, one of which was reduced with 10 mM dithiothreitol before $NaDodSO_4$/gel electrophoresis.

Immunoprecipitation of Radiolabeled Partially Purified Material. Radiolabeled partially purified material (3×10^6 cpm) or ^{125}I-PDGF (10^5 cpm) was incubated with 10 μl of anti-PDGF immune serum, or a nonimmune serum, in a total volume of 30 μl for 4 hr at 4°C. Protein A Sepharose (Pharmacia) (20 μl of packed beads in a volume of 40 μl) was then added, and incubation was prolonged for another 30 min at 4°C. After washing 4 times in radioimmunoassay washing buffer (see above) and 1 time in 10 mM phosphate buffer (pH 7.4), protein A Sepharose-bound radioactivity was released by incubation for 3 min at 95°C in 3.6% $NaDodSO_4$/80 mM Tris·HCl, pH 8.8/0.01% bromophenol blue. The samples were then divided in two equal parts, one of which was reduced with 10 mM dithiothreitol before $NaDodSO_4$/gel electrophoresis.

Polyacrylamide Gel Electrophoresis in $NaDodSO_4$. Gel electrophoresis in $NaDodSO_4$ was carried out as described (27), using 13–18% acrylamide gradient gels. Gel dimensions were 300 × 200 × 1 mm and gels were run overnight at a constant current at room temperature. After electrophoresis, gels were stained for protein, dried, and subjected to autoradiography (28).

RESULTS

PDGF Receptor Competing Activity in Glioma Conditioned Medium. Serum-free conditioned medium was harvested from confluent cultures of U-343 MGa Cl 2 cells after 3 days of incubation and analyzed with regard to competition with ^{125}I-PDGF for binding to the PDGF receptor (Fig. 1). A dose-dependent PDGF receptor competing activity was found; its concentration in the harvested medium was equivalent to 20–30 ng of PDGF per ml. The cumulative production of competing activity was essentially linear with time over a period of 18 days and virtually parallel to the accumulation of protein in the extracellular medium (Fig. 2).

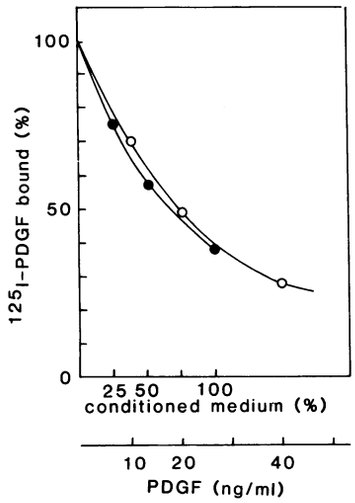

FIG. 1. Effect of conditioned medium from a human clonal glioma cell line on ^{125}I-PDGF binding to human foreskin fibroblasts. The cell line U-343 MGa Cl 2 was cultured to confluence and transferred to serum-free F-10; medium was harvested after 3 days of incubation. The medium was assayed for PDGF receptor competing activity. A competing activity was shown in the glioma medium closely paralleling the displacing activity of unlabeled purified PDGF. ○, PDGF; ●, conditioned medium.

Properties of Partially Purified Glioma Factor. The PDGF receptor competing activity of conditioned serum-free medium (6 liters) from U-343 MGa Cl 2 cultures was concentrated by chromatography on Sulphadex beads and then chromatographed on Sephadex G-200 (Fig. 3). ^{125}I-PDGF competing activity migrated as a single broad peak after the major protein components; it had an elution position close to that of ^{125}I-PDGF. The estimated amount of recovered competing activity corresponded to 8 μg of PDGF. Growth promoting activity was found in the same region in the chromatogram; it emerged as a broad peak entirely overlapping the ^{125}I-PDGF peak and the peak of PDGF receptor competing activity. Material crossreacting with PDGF in the radioimmunoassay showed similar chromatographic behavior as the receptor-binding activity but occurred in far lower amounts; in terms of PDGF equivalents, immunological crossreactivity was only ≈1% of the receptor-binding activity.

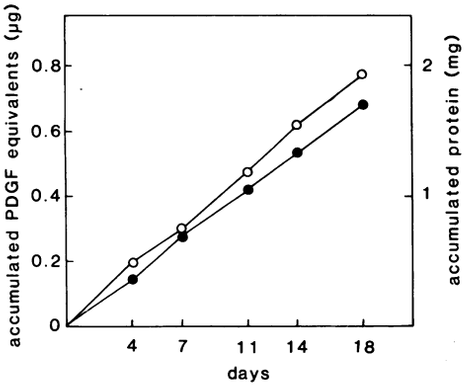

FIG. 2. Cumulative production of a PDGF analog by U-343 MGa Cl 2 glioma cells. Cells were cultured in 50-mm Petri dishes, grown to confluence in serum-containing medium, and then maintained in serum-free F-10 medium. Media were harvested every 3 or 4 days and PDGF receptor competing activity was measured on AG 1523 fibroblasts and converted to PDGF equivalents using a standard curve obtained with pure PDGF. ○, Protein; ●, PDGF equivalents.

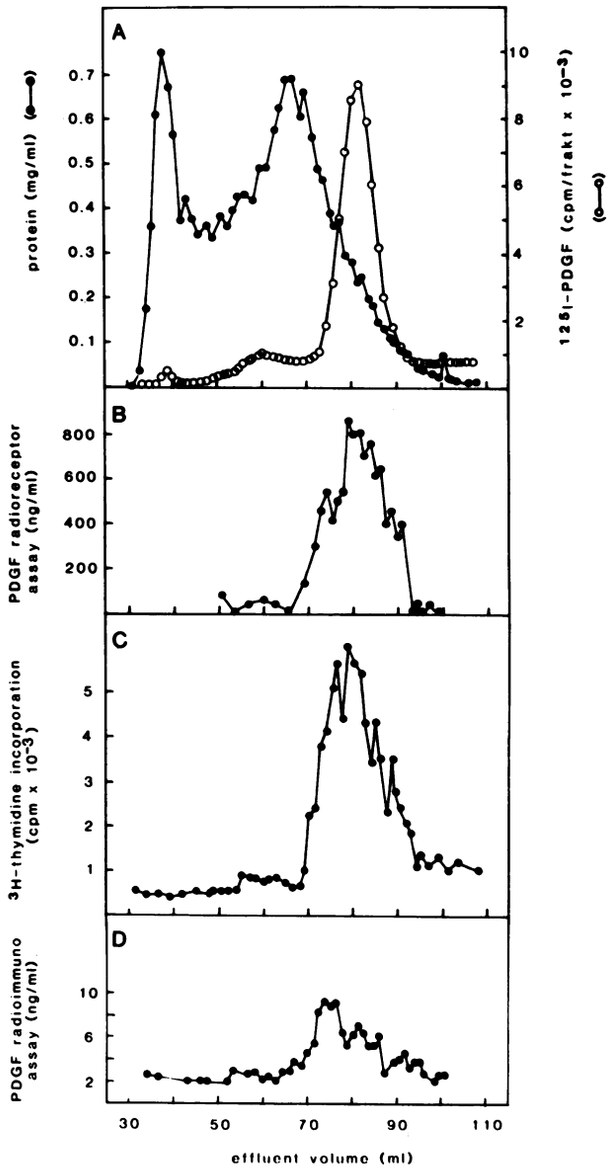

FIG. 3. Chromatography on Sephadex G-200 of conditioned medium from the glioma cell line U-343 MGa Cl 2. Effluent fractions were analyzed for protein (*A*), activity competing with ^{125}I-PDGF for binding to fibroblast receptors (*B*), growth-promoting activity (*C*), activity competing with ^{125}I-PDGF for binding to PDGF antibodies (*D*). The elution position of ^{125}I-PDGF is indicated in *A*.

Binding to Fibroblast Cultures. The results above show that U-343 MGa Cl 2 cells release a factor(s) with certain PDGF-like properties. Additional support for this finding was derived from the analysis of ^{125}I-labeled partially purified material. Incubation of this preparation with fibroblast cultures led to the selective binding of a single component with an estimated M_r of 31,000 as shown by NaDodSO$_4$/gel electrophoresis under nonreducing conditions (Fig. 4). Binding of this component did not occur in the presence of excess unlabeled PDGF, indicating that it involved the PDGF receptor in a specific manner. After reduction, the bound component appeared as a single band with a higher electrophoretic mobility, indicating a M_r of 18,000. It was concluded that the ^{125}I-labeled preparation of glioma factor contained a PDGF-like component having similar receptor-binding and molecular weight properties as authentic PDGF.

Recognition by PDGF Antibodies. Incubation of the ^{125}I-labeled material with an antiserum raised against PDGF and

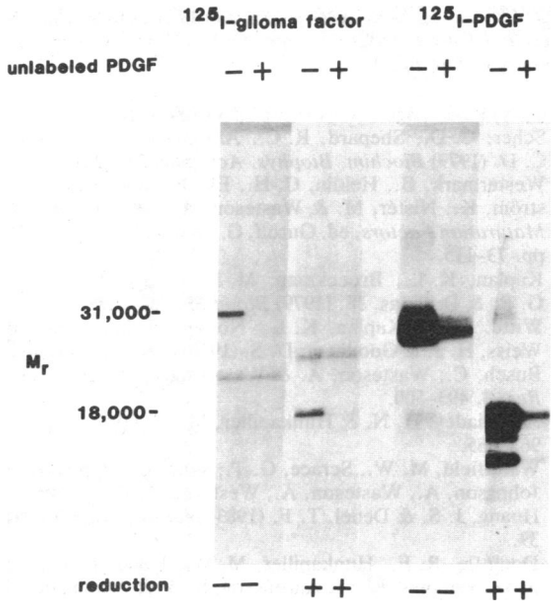

FIG. 4. Adsorption to human AG 1523 fibroblasts of ^{125}I-PDGF or ^{125}I-labeled partially purified glioma factor. Binding was carried out in the absence or presence of a 50-fold molar excess of unlabeled PDGF. The adsorbed material was analyzed by NaDodSO$_4$/gel electrophoresis; radioactivity was visualized by autoradiography.

subsequent exposure to Protein A Sepharose caused precipitation of a significant portion of the radioactive material. In contrast, precipitation with control serum yielded only little radioactivity. Analysis by NaDodSO$_4$/gel electrophoresis revealed that the immunoprecipitated material behaved like a single M_r 31,000 component (Fig. 5). Under reducing conditions it attained the mobility of a M_r 18,000 component. In conclusion, a PDGF-like species was among the ^{125}I-labeled glioma products; it was specifically recognized by PDGF antibodies and had similar electrophoretic characteristics as PDGF proper.

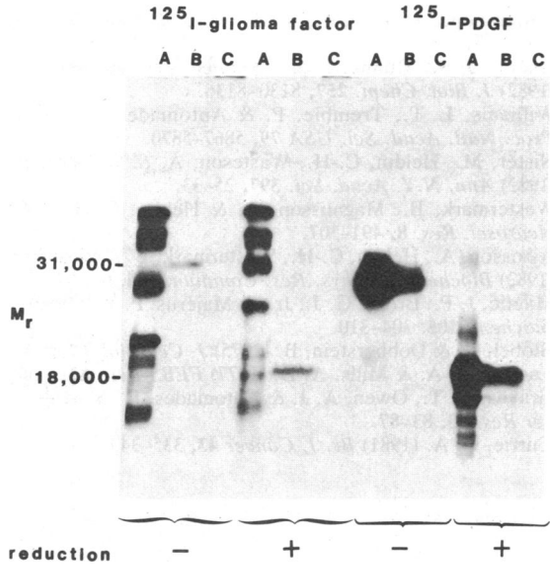

FIG. 5. Immunoprecipitation of ^{125}I-PDGF or ^{125}I-labeled partially purified glioma factor. Precipitated material was analyzed by NaDodSO$_4$ gel electrophoresis; radioactivity was visualized by autoradiography. Lanes: A, starting material; B, precipitation with PDGF antiserum; C, precipitation with control serum.

Table 1. Adsorption of PDGF receptor competing activity to immobilized PDGF antibodies

	PDGF receptor competing activity remaining in supernatant (ng of PDGF equivalents per ml)	
	Control beads	Beads substituted with PDGF antibodies
PDGF	700	20
Glioma factor	1,100	370

Serum-free medium conditioned by 343 MGa Cl 2 cells was harvested and ^{125}I-PDGF competing activity was concentrated on Sulphadex beads. This material, or for comparison pure PDGF, was mixed with PDGF antibodies immobilized on Sepharose (250 μl of packed beads; 5 mg of the immunoglobulin fraction from the anti-PDGF immune serum was coupled to 1 ml of CNBr-activated Sepharose) in a total volume of 1 ml of phosphate-buffered saline, incubated under shaking overnight, and then centrifuged. The supernatants were analyzed with regard to their effect on ^{125}I-PDGF binding to human fibroblasts. For controls, glioma factor or PDGF was mixed with a similar amount of uncoupled Sepharose beads.

An analogous experiment was carried out with unlabeled glioma factor to examine whether PDGF-antibodies similarly recognized PDGF receptor competing activity. A preparation of glioma factor that had been passed over a Sulphadex column was incubated overnight with Sepharose 4B beads to which PDGF antibodies had been coupled. This treatment markedly decreased the amount of PDGF receptor competing activity remaining in the supernatant (Table 1). A similar effect was noticed in experiments with pure PDGF rather than glioma factor, whereas, in contrast, plain Sepharose beads did not decrease PDGF receptor-competing activity in either case.

DISCUSSION

The present data show that serum-free conditioned medium from the human clonal glioma cell line U-343 MGa Cl 2 blocks the binding of ^{125}I-PDGF to human foreskin fibroblasts. Several lines of evidence show that this activity is exerted via the binding of a PDGF-like factor to the cellular PDGF receptor. Most compelling evidence was derived from experiments using ^{125}I-labeled partially purified material; both fibroblasts, through their PDGF receptor, and PDGF antibodies selectively recognized a M_r 31,000 component which upon reduction migrated as a M_r 18,000 species on NaDodSO$_4$/gel electrophoresis. According to the model recently proposed (25), PDGF purified from human platelets consists of two polypeptide chains, designated A and B, which are linked by disulfide bonds. Isolated A chain appears as multiple bands on NaDodSO$_4$/gel electrophoresis in the molecular weight range 18,000–11,000, whereas the B chain migrates as a single M_r 16,000 species. The heterogeneity in molecular weight of unreduced PDGF (25) is thus most probably due to a partial degradation of the A chain, possibly due to a proteolytic cleavage during the course of preparation. Iodination of PDGF by the chloramine-T method causes the preferential labeling of the A chain (unpublished observation). Consequently, when ^{125}I-PDGF is electrophoresed under reducing conditions, the B chain does not appear on the autoradiograms. The molecular weight of the glioma-derived factor of the present investigation seems to correspond to a large (M_r 31,000) form of PDGF. Moreover, the ^{125}I-labeled component recognized by cells and PDGF antibodies behaves after reduction as expected from a PDGF of this molecular weight—i.e., it migrates as a single M_r 18,000 species corresponding to a nondegraded A chain.

An immunological crossreactivity of the glioma-derived analog with PDGF was shown by three experimental procedures using PDGF antiserum: immunoprecipitation of ^{125}I-labeled material as discussed above, absorption of PDGF re-

ceptor binding activity by immobilized PDGF antibodies, and activity in the PDGF radioimmunoassay. Although the glioma-derived factor thus is immunologically related to PDGF, the two factors do not seem to be identical because the crossreactivity in the radioimmunoassay did not match the ^{125}I-PDGF competing activity in the receptor binding assay. However, when an excess of PDGF antibodies was used, a major part of the competing activity of the glioma factor was precipitated (Table 1). The possibility that a minor part of the glioma factor activity is identical to PDGF and a major part unrelated is thus unlikely. Rather, these observations support the possibility that the PDGF antibodies recognize all glioma cell produced activity, but at a lower affinity. More detailed information is required, however, to determine if the glioma-derived analog is a different gene product than PDGF or represents a post-translationally modified factor. It is noteworthy that TGF produced by virus transformed cells is functionally related to EGF in the sense that it displaced EGF from the EGF receptor but seemingly is immunologically unrelated (11).

The fact that the growth-promoting activity of the glioma factor preparation comigrated with authentic PDGF on Sephadex G-200 chromatography supports the idea that this activity should similarly be ascribed to a PDGF-like molecule. Although this has not been rigorously proven, it is reasonable considering the presence of PDGF-receptor binding material in the same fractions. The glioma-derived growth factor is thus another example of a tumor cell-derived analog to PDGF; previously, PDGF-like properties have been assigned to an osteosarcoma-derived growth factor first described by our group (15) and recently confirmed by Graves *et al.* (29).

The current interest in tumor-derived growth factors relates to the idea that tumor cells may use endogenous growth factors in an autocrine manner—i.e., to stimulate the proliferation of the producer cell itself. In addition, some of these factors interestingly have transforming activity (TGFs)—i.e., ability to cause a reversible phenotypical transformation of normal cells (11–13). The recently described amino acid homology between the putative transforming protein of simian sarcoma virus, p28^{sis}, and PDGF suggests that PDGF may be encoded by the normal cellular homologue to v-*sis* (c-*sis*). The structural, immunological and functional similarity between the osteosarcoma (15) and glioma-derived growth factors and PDGF therefore suggests that these tumor-derived factors are also derived from the *sis* gene family. Interestingly, PDGF seems to act as a transforming growth factor, because it causes an irregular (criss-cross) growth pattern in BALB/c 3T3 cultures (3) and growth in agar of untransformed BHK cells (30). In addition, both PDGF and the glioma factor induce anchorage-independent growth of nontransformed Rat 1 cells (unpublished observations). Assuming that the PDGF analog of osteosarcoma and glioma cells may also be manufactured *in vivo*, one may speculate that the autocrine activation of the PDGF receptor may be operational in the growth of these human neoplasms.

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