Vol. 117, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1983 Pages 176-182

SYNTHESIS OF A PDGF-LIKE GROWTH FACTOR IN HUMAN GLIOMA AND SARCOMA CELLS SUGGESTS THE EXPRESSION OF THE CELLULAR HOMOLOGUE TO THE TRANSFORMING PROTEIN OF SIMIAN SARCOMA VIRUS

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Received October 12, 1983

SUMMARY: Several human normal and neoplastic cell lines were screened for production of PDGF receptor competing activity. Conditioned medium from two sarcomas and one glioma blocked ^{125}I-PDGF binding to human foreskin fibroblasts in a dose-dependent manner. In each case this effect was abolished when the conditioned medium was pretreated with PDGF-antiserum, indicating that the receptor competing activity was immunologically related to PDGF. Direct evidence for *de novo* synthesis of a PDGF-like component in the cultures was afforded by ^{35}S-cysteine labeling of the three cell lines, followed by immunoprecipitation with PDGF antiserum. This resulted in the specific precipitation of a 31,000 molecular weight labeled protein, which upon reduction was split into two polypeptides of molecular weights 17,000 and 16,500. The significance of these findings in view of the recently discovered structure homology between PDGF and the transforming gene product of simian sarcoma virus, p28^{sis}, is discussed.

Platelet-derived growth factor (PDGF) is a potent mitogen for a variety of cultured cells of mesenchymal and glial origin (reviewed in ref. 1-3). A partial amino acid sequence analysis of human PDGF has revealed a striking homology with the transforming protein of simian sarcoma virus, p28^{sis} (4-6). This finding suggests that simian sarcoma virus has obtained transforming capacity by acquiring cellular PDGF sequences. Moreover, the structural homology between p28^{sis} and PDGF suggests a similar homology in functional properties, i. e. p28^{sis} may excert its transforming activity by interacting with the PDGF receptor.

Transfection experiments using DNA from tumor cell lines have recently provided evidence for the involvement of oncogenes in human malignancies

ABBREVIATIONS: PDGF, platelet-derived growth factor; Eagle's MEM, Eagles minimal essential medium.

0006-291X/83 $1.50

(7,8). The most commonly used target cells for transfection, the NIH 3T3 cell line seems, however, restricted in their transforming ability since virtually only members of the ras gene family have been identified in this assay. The striking similarity between the v-sis gene product and PDGF opens the possibility to search for the expression of sis-related sequences in human tumor cell lines, using specific assay methods previously established for PDGF. The present study presents direct evidence that two human sarcoma and one human glioma cell lines synthesize and release PDGF, or a closely related molecule, which may be the cellular homologue to $p28^{sis}$.

MATERIALS AND METHODS

Purification and radiolabeling of PDGF

PDGF was prepared from human platelets (9) and radiolabeled (10) as described. An initial specific activity of about 30,000 cpm/ng was obtained.

Harvest of conditioned media

For the harvest of conditioned media, several different human tumor cell lines as well as normal cells were first grown to confluence on Falcon 60 cm^2 dishes or in Falcon 850 cm^2 plastic roller bottles in Eagle's MEM supplemented with 10% newborn calf serum. Cultures were then washed 3 times with phosphate-buffered saline and fed with serum-free MCDB 104 medium (11) or a mixture of Eagle's MEM and MCDB 104 (1:1). Medium was collected and cells were refed every 48 h. The cells were maintained in serum-free culture for up to 14-30 days.

PDGF receptor competition assay

The PDGF receptor competition assay was performed as described (10). Briefly, confluent cultures (4.5 cm^2) of human foreskin fibroblasts (AG 1523, obtained from the Human Genetic Mutant Cell Repository, Camden, NJ) were washed once in washing buffer (phosphate buffered saline, containing 0.9 mM $CaCl_2$ and 0.8 mM $MgSO_4$, supplemented with 1 mg of human serum albumin per ml) and then exposed to various concentrations of conditioned media, or conditioned media concentrated five times using a Minicon M15 concentrator, for 2 h at 4°C. Then the cultures were washed three times in washing buffer and incubated for another 1 h with ^{125}I-PDGF (10 ng/ml). Cultures were then washed 5 times in washing buffer and cell-bound radioactivity collected after solubilization with 1% Triton X-100, 10% glycerol, 20 mM HEPES pH 7.4 and 1 mg/ml human serum albumin for 20 min at room temperature.

Biosynthetic labeling, immunoprecipitation, and gel electrophoresis

Cells were grown to confluence at 37°C in Eagle's MEM supplemented with 10% newborn calf serum in 850 cm^2 Falcon roller bottles. Cells were then grown in serum-free MCDB 104 medium for one week with two medium changes and then given 20 ml of cysteine-free MCDB 104 medium supplemented with 1 mCi of ^{35}S-cysteine (600 Ci/mmol, New England Nuclear). After incubation for 36 h at 37°C, the medium was harvested and exposed to 50 ul of a rabbit control serum. After incubation for 20 h at 4°C, Protein A-Sepharose (Pharmacia) was added (100 ul of packed beads) and incubation prolonged for 2 h. The beads were then collected by centrifugation and the supernatants each received 50 ul of anti PDGF immune serum (12). After incubation for 20 h at 4°C, immune complexes were adsorbed to Protein A-Sepharose as described above. The Protein A-Sepharose beads were washed 4 times in the centrifuge with 0.5 M NaCl, 0.01 M

phosphate buffer pH 7.4, 0.5% bovine serum albumin, 0.1% Tween 80 and then one additional time in 10 mM Tris buffer pH 7.4. The beads were then given 100 ul of 3.6% SDS, 80 mM Tris buffer pH 8.8, 0.01% bromophenol blue and heated at 95°C for 3 min. The supernatants were divided in two equal portions, one of which was reduced by incubation with 10 mM dithiothreitol for three min at 95°C, and analyzed by SDS-gel electrophoresis using 13-18% gradient gels (13). For comparison, immunoprecipitated ^{125}I-PDGF was also run on the gel. After electrophoresis the gel was fixed for 30 min in 10% trichloroacetic acid, stained for protein with Coomassie Brilliant Blue R-250, destained and incubated for 1 h in EN^{3}HANCE (New England Nuclear) followed by 30 min in distilled water. The gel was then dried (14) and subjected to fluorography using Fuiji RX films and exposure at -70°C for 3-7 days. As standards for molecular weight determination were used the following reduced proteins: Phosphorylase b (molecular weight 94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soy bean trypsin inhibitor (20,100) and α-lactalbumin (14,400).

RESULTS

Serum-free, conditioned media from several human cell lines of normal and neoplastic origin were screened for the presence of factors competing with ^{125}I-PDGF binding to human fibroblasts. Out of 27 lines analysed to date (4 sarcomas, 16 gliomas, 2 neuroblastomas, 2 carcinomas, 1 normal human fibroblast line and 2 normal glial cell lines), three lines have appeared positive: an osteosarcoma cell line (U-2 OS) (15), a glioma cell line (U-343 MGa clone 2) (16) and a rhabdomyosarcoma cell line (RD) (17). Conditioned medium from any of these three lines inhibited binding of ^{125}I-PDGF to fibroblasts in a dose-dependent manner (Fig 1). The concentration of PDGF-receptor competing activity in the media corresponded to 5-20 ng/ml of PDGF. The results obtained with two non-producing human tumor cell lines (U-393 OS (18) and A 431 (19)) and a normal human fibroblast cell line are also shown. The PDGF-receptor competing activity seems immunologically related to PDGF since it was inhibited by pretreatment of the culture medium with PDGF antiserum, whereas a control rabbit serum was without effect (Table 1).

The producer cell lines as well as two non-producing lines (U-393 OS and AG 1523) were selected for metabolic labeling with ^{35}S-cysteine. A 31 kDa component was selectively precipitated by anti PDGF serum from the culture media of U-2 OS, U-343 MGa Clone 2 and RD. In some cases, additional components were nonspecifically precipitated by anti PDGF serum as well as control serum. In media from the two cell lines that did not produce PDGF receptor

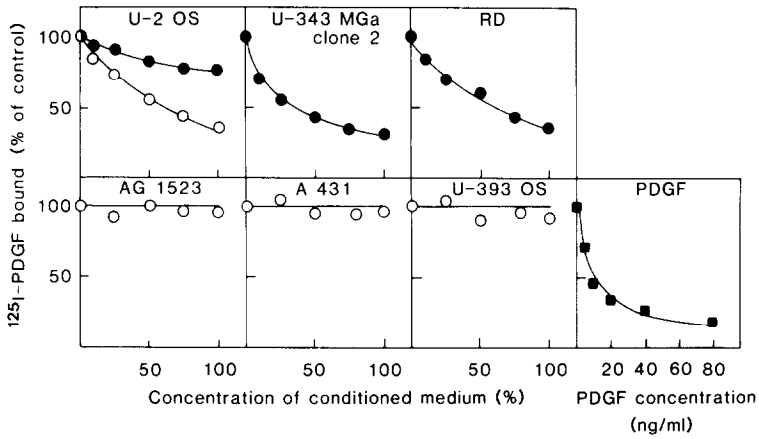

Fig. 1. Analysis of PDGF receptor competing activity in culture media from human cell lines. Conditioned media from different cell lines were collected and subjected to the PDGF receptor competition assay, directly (0-0) or after concentration 5 times (0-0), as described in Materials and Methods. Cell-bound radioactivity is expressed as per cent of controls which received no conditioned medium during incubation. The controls bound 15-20 x 10^3 cpm per 210-250 x 10^3 cells. A standard curve showing the competing activity of unlabeled PDGF is also given.

competing activity, no components were specifically precipitated by the anti PDGF serum (Fig 2). The metabolically labeled products had homogenous motilities, similar to that of the most high-molecular weight component of ^{125}I-PDGF (Fig 2); as expected ^{125}I-PDGF produced a number of peptides in the range 28 kDa to 31 kDa, probably representing degradation of PDGF during purification

Table 1. Effect of PDGF-antiserum on PDGF receptor competing activity in cell culture conditioned media.

	^{125}I-PDGF bound (% of control)		
Origin of medium	no serum	normal rabbit serum	PDGF antiserum
U-2 OS	74	78	101
U-343 MGa Cl 2	48	54	87
RD	42	48	91

Conditioned media were incubated with either a normal rabbit serum or PDGF antiserum. Immune complexes were then removed by adsorbtion to protein A-Sepharose as described for the ^{35}S-cysteine labeling experiments in Materials and Methods. The non-precipitated fractions were then subjected to the PDGF receptor competition assay. Cell bound radioactivity is expressed as per cent of a control which received no conditioned medium during incubation (7400 cpm per 230 x 10^3 cells).

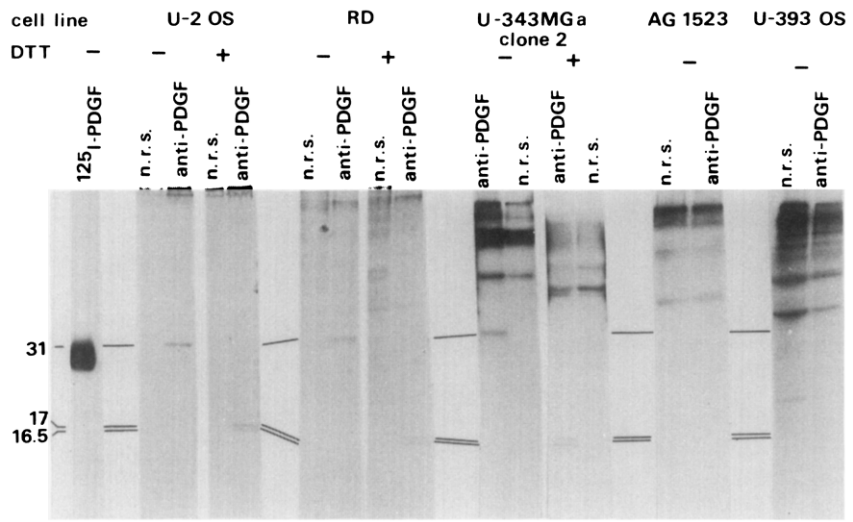

Fig. 2. Immunoprecipitation of a PDGF-like substance from the medium of U-2 OS, U-343 MGa clone 2 and RD cells after biosynthetic labeling using ^{35}S-cysteine. Biosynthetic labeling, immunoprecipitation and analysis by SDS-gel electrophoresis followed by fluorography, was performed as described in Materials and Methods.

(20-23). Thus, the ^{35}S-cysteine-labeled products behaved like the least degraded species of ^{125}I-PDGF. After reduction, each of the 31 kDa components were converted to two faster migrating polypeptides of molecular weights 17,000 and 16,500. Thus, the metabolically labeled components from different tumor cell lines are mutually similar or identical and probably composed of two disulphide linked polypeptide chains; this type of structure has previously been shown for the PDGF molecule (8).

DISCUSSION

The data presented in this communication provides direct evidence that certain human sarcoma and glioma cell lines synthesize and secrete PDGF, or a closely related substance. We have previously presented circumstantial evidence for the presence in culture media from U-2 OS (24) and U-343 MGa clone 2 (18) of a growth factor(s) with physico-chemical properties in common with PDGF. Another factor which may be related to PDGF has also been isolated from SV-40 transformed BHK cells (25,26).

The recently described amino acid sequence homology between human PDGF and p28^{sis} suggests that PDGF is the normal cellular homologue to the transforming

protein of simian sarcoma virus. Available data suggests that PDGF in vivo is synthesized by megakaryocytes, stored and transported by platelets and released during platelet activation to play a role in tissue repair. Thus, under normal circumstances, cells are probably not exposed to PDGF. The unscheduled synthesis and release of PDGF or a functionally related molecule by tumor cells may lead to an uncontrolled activation of the PDGF receptor and continuous growth stimulation. The finding of PDGF production in osteosarcoma, rhabdomyosarcoma and glioma cells is of particular significance since expression of the PDGF receptor seems to be restricted to cells of mesenchymal or glial origin. In addition, v-sis related transcripts have been found at a high frequency in human glioma and sarcoma cells but not in human carcinoma cells or normal fibroblasts (27). Support for the idea that endogenously produced growth factors may stimulate growth in an autocrine manner is derived from the recent demonstration of PDGF receptors on cloned U-2 OS cells and the presence of PDGF-dependent kinase activity in membranes prepared from these cells (Betsholtz et al., unpublished).

It is generally believed that carcinogenesis in vivo is a multistep phenomenon involving several genetic loci. In analogy with this assumption it has recently been postulated that several complementary oncogenes have to be expressed to cause a complete transformation (28,29). Experimental support for this idea has been obtained from experiments where primary rat embryo fibroblasts have been transformed by various pairs of oncogenes (28,29). In view of this finding, the demonstration of a PDGF-like biosynthetic product in RD cells is of particular interest since DNA from this cell line contains transforming sequences of the ras gene family (30,31). The neoplastic properties of the RD cells may thus be the result of the concerted action of at least two oncogenes, belonging to the ras and sis gene families, respectively.

ACKNOWLEDGMENTS

These studies were supported by grants from the Swedish Cancer Society (689, 786 and 1794), the Swedish Medical Research Council (4486), Konung Gustaf V:s åttioårsfond and the University of Uppsala.

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