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Human melanoma cell lines of primary and metastatic origin express the genes encoding the chains of platelet-derived growth factor (PDGF) and produce a PDGF-like growth factor

(*SIS*/A chain/pigmented lesion)

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ABSTRACT **Normal human melanocytes and five human melanoma cell lines were analyzed for production of platelet-derived growth factor (PDGF)-like activity. Three of the melanoma cell lines released an activity that inhibited binding of ^{125}I-labeled PDGF to human foreskin fibroblasts and stimulated [^{3}H]thymidine incorporation in such cells. These activities were inhibited by the addition of anti-PDGF antibodies. All three factor-producing cell lines were derived from the same patient—one originated from the primary tumor (WM 115), and two were from individual lymph-node metastases (WM 239A and WM 266-4). The factor produced by WM 266-4 cells was characterized biochemically in detail. Immunoprecipitated, the metabolically labeled factor migrated in NaDodSO$_4$/gel electrophoresis as a homogeneous M_r 31,000 species, which under reducing conditions was resolved into two species of M_r 16,500 and M_r 17,000, implying a dimeric structure of the molecule. The factor was purified to homogeneity. Analysis by reverse-phase high-pressure liquid chromatography of reduced and alkylated factor revealed an elution pattern identical to that of PDGF A chains. Thus, the native molecule appears to be a homodimer of PDGF A chains. Blot-hybridization analysis of poly(A)$^+$ RNA from the cell lines with ^{32}P-labeled PDGF A chain and B chain (*SIS* product) cDNA probes revealed a relative abundance of B chain transcripts in the cell line originating from the primary tumor tissue only but expression of A chain in all three cell lines. We conclude that the two structural genes encoding each of the subunit chains of PDGF can be expressed in human melanoma cells and that the two genes can be independently expressed in such cells.**

Human platelet-derived growth factor (PDGF) is a potent mitogen for a variety of cells in culture (1, 2). The factor is a M_r 30,000 dimer of A and B chains linked by disulfide bonds (3). The amino acid sequence of the B chain is virtually identical to that of part of p28^{sis}, the product of the oncogene v-*sis* of simian sarcoma virus (4–10). The complete amino acid sequence of the A chain precursor has been deduced from the nucleotide sequence of cloned cDNA (11) and, within the parts corresponding to the mature chains, it shows nearly 60% homology to the PDGF B chain.

Simian sarcoma virus transformation appears to be mediated through an autocrine mechanism (12, 13). Involvement of PDGF in human malignancy is suggested by the findings of expression of *SIS* (human gene designation for c-*sis*, the cellular homolog of v-*sis*) and/or production of PDGF-like activity in a number of human tumor cell lines, including those of neural crest origin (14–18). In the present investigation, we analyzed cell lines derived from human malignant melanoma, a neural crest-derived malignancy (19), for production of PDGF-like activity and expression of the two genes that encode the subunit chains of PDGF. We demonstrate here that PDGF-like activity is produced by three cell lines: one derived from a primary melanoma and two from metastases of the same patient. Moreover, we show that all three cell lines contain a relative abundance of PDGF A chain transcripts, whereas only the primary-tumor cell line expresses a high level of *SIS* mRNA—i.e., B chain transcripts.

MATERIALS AND METHODS

Cell Culture. Human normal melanocytes were isolated and cultured as described (20). The establishment and *in vitro* characteristics of the human melanoma cell lines WM 9, WM 115, WM 239A, and WM 266-4 have been described (21). The melanoma cell line SW 691 was obtained from A. Leibovitz. Human foreskin fibroblasts (AG 1523) were purchased from the Human Mutant Cell Repository, Institute for Medical Research (Camden, NJ). Cells were routinely grown 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 passaged twice a week by using 0.2 mg of EDTA and 2.5 mg of trypsin (Difco) per ml of phosphate-buffered saline (8 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4, and 1.15 g of Na_2HPO_4 (anhydrous) per liter for detachment of the monolayer.

Confluent cultures were washed once with serum-free nutrient medium F-10 and incubated in this medium for 3 days (1–2 ml of medium per 10^5 cells), and the medium was harvested. Medium was then centrifuged at 10,000 × *g* for 10 min and stored at −20°C until use. For the production of a large volume of conditioned medium, WM 266-4 cells were grown in Falcon plastic roller bottles (850 cm^2) rotated at 0.5 rpm.

PDGF and PDGF Antibodies. PDGF was purified from fresh human platelets (22) and labeled with ^{125}I by the chloramine-T method to a specific activity of about 50,000 cpm/ng (23). Immunoglobulin fractions from a rabbit anti-PDGF antiserum (24) were isolated by affinity chromatography on protein A-Sepharose (Pharmacia).

Assay of Growth-Promoting Activity. Growth-promoting activity was measured as stimulation of [^{3}H]thymidine incorporation in serum-free cultures of human foreskin fibroblasts maintained in MCDB 104 medium at reduced calcium con-

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Abbreviations: PDGF, platelet-derived growth factor.

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centration (0.5 mM) (25). Radioactivity incorporated into trichloroacetic acid-precipitable material was measured by liquid scintillation.

Assay for PDGF Receptor-Competing Activity. PDGF receptor-competing activity in conditioned medium was measured as the inhibition of ^{125}I-labeled PDGF (^{125}I-PDGF) binding to confluent cultures of foreskin fibroblasts (16). Incubation with conditioned medium was performed for 2 hr at 4°C. The cultures were then washed four times with binding medium (phosphate-buffered saline containing 1 mg of human serum albumin, 0.01 mg of $CaCl_2 \cdot 2H_2O$, and 0.01 mg of $MgSO_4 \cdot 7H_2O$ per ml) and analyzed with regard to ^{125}I-PDGF binding. An immunological crossreactivity with PDGF was analyzed by adding 40 μg of anti-PDGF IgG or nonimmune IgG to the conditioned medium during the preincubation period. Competing activity was converted to PDGF equivalents (ng/ml) by using a standard curve constructed from results obtained with known concentrations of pure PDGF (5–40 ng/ml).

Assay for ^{125}I-PDGF Binding. Cells were grown to confluence in 12-well cluster plates (4.2 cm^2 per well) and washed once with binding medium. Cultures were incubated at 4°C for 2 hr in 0.5 ml of binding medium containing ^{125}I-PDGF (2 ng/ml) with or without a 100-fold excess of unlabeled PDGF. Cell-associated radioactivity was sampled and measured as described (23).

Metabolic Labeling of Melanoma Cells. Confluent cultures of cell line WM 266-4 were incubated with [^{35}S]cysteine (0.5 mCi/ml; 600 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) for 3 hr in cysteine-free, serum-free Eagle's minimum essential medium, followed by a 2-hr chase in medium containing unlabeled cysteine. This medium was harvested and sequentially immunoprecipitated with control rabbit serum or PDGF antiserum (15, 24). Immune complexes adsorbed to protein A-Sepharose were analyzed by NaDodSO$_4$/gel electrophoresis under reducing (10 mM dithiothreitol) and nonreducing conditions with 13–18% acrylamide gradient gels (26). Electropherograms were examined by fluorography (27).

Purification and Radioiodination of Melanoma-Derived Growth Factor. The PDGF-like growth factor was purified from serum-free medium conditioned by WM 266-4 melanoma cells, following the protocol used for the purification of osteosarcoma-derived growth factor (28). Briefly, the factor was first adsorbed to a highly charged Sulphadex gel, eluted with NaCl, and subjected to two gel chromatographic analyses—one at neutral pH and one at low pH. Final purification was achieved by reverse-phase HPLC. The purified factor was ^{125}I-labeled by the method of Bolton and Hunter (29).

Preparation of RNA and Blot-Hybridization Analysis. Total cellular RNA was isolated by using the LiCl/urea method (30). Poly(A)$^+$ RNA was selected by chromatography on oligo(dT)-cellulose (Pharmacia). Agarose gel electrophoresis, blotting to nitrocellulose filters, and hybridization to nick-translated probes were carried out by standard techniques (31). The probes used were a 2.7-kilobase human *SIS* cDNA fragment cloned in a pBR322 derivative (pSM1, a generous gift from Flossie Wong-Staal) and a 1.3-kilobase human PDGF A chain cDNA cloned in pUC-13 (13). Exposure to Kodak XAR-5 films was at −70°C for 4 days.

RESULTS

PDGF Receptor-Competing Activity in Melanoma-Conditioned Medium. Serum-free conditioned media from cultures of normal melanocytes, five melanoma cell lines, and human foreskin fibroblasts were analyzed as described for the ability to compete for ^{125}I-PDGF binding to the PDGF receptor on human foreskin fibroblasts (Table 1). Three of the melanoma lines, originating from the primary tumor (WM 115) and two individual metastases (WM 239A and WM 266-4) from the same patient, showed significant activity, whereas no detectable activity was observed in the medium of the other cell lines tested. None of the melanoma cell lines displayed any specific binding of ^{125}I-PDGF, in contrast to foreskin fibroblasts, which were included as a control. WM 266-4 cells were selected for further characterization of the melanoma-derived PDGF-like factor. Addition of rabbit anti-PDGF IgG (40 μg/ml) markedly inhibited PDGF receptor-competing activity (Fig. 1 *Upper*) and mitogenic activity (Fig. 1 *Lower*) in cultures of foreskin fibroblasts.

Table 1. Production of PDGF receptor-competing activity and binding of ^{125}I-PDGF to human melanoma cells and foreskin fibroblasts

Cell line	Tissue origin	PDGF receptor-competing activity, ng/ml	^{125}I-PDGF binding, fmol per 10^6 cells
WM 9	Primary melanoma	<5	<0.2
WM 115*	Primary melanoma	37	<0.2
WM 239A*	Metastatic melanoma	32	<0.2
WM 266-4*	Metastatic melanoma	32	<0.2
SW 691	Primary melanoma	<5	<0.2
	Normal melanocytes	<5	†
AG 1523	Foreskin	<5	11.4

*Cell lines originated from the same patient.
†A low ^{125}I-PDGF binding was obtained. Whether this reflects contamination with a small percentage (<1%) of fibroblasts or represents a low number of PDGF receptors is not known.

Biochemical Analysis of Melanoma-Derived Growth Factor.

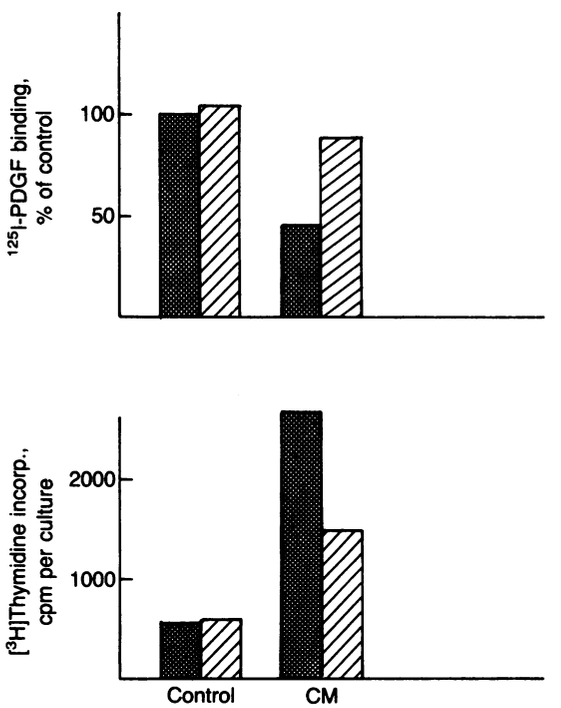

FIG. 1. PDGF-like activity in conditioned medium (CM) from melanoma cells. Serum-free CM was harvested from confluent cultures of melanoma WM 266-4 cells and assayed for PDGF receptor-competing activity (*Upper*) and growth-promoting activity (*Lower*) in cultures of human foreskin fibroblasts as described. Incubations were done in the presence of nonimmune rabbit IgG (stippled bars) or anti-PDGF IgG (hatched bars). In *Upper* the antibodies were present during a preincubation period of 2 hr as specified in *Materials and Methods*, whereas in *Lower* the antibodies were present during the entire assay period of 48 hr (25). Incorp., incorporation.

Analysis by NaDodSO$_4$/gel electrophoresis of ^{35}S-labeled material immunoprecipitated from WM 266-4 melanoma cell supernatant with PDGF antibodies revealed a single, homogeneous component of M_r 31,000 under nonreducing conditions and two faster-migrating species of M_r 17,000 and M_r 16,500 under reducing conditions (Fig. 2). This pattern of migration is identical to that of the previously described osteosarcoma-derived growth factor (28).

The melanoma-derived growth factor was purified from 3 liters of WM 266-4 conditioned medium, yielding about 10 μg of final product. Analysis by NaDodSO$_4$/gel electrophoresis of ^{125}I-labeled material indicated a homogeneous component of M_r 31,000, which was converted to M_r 17,000 after reduction (not shown). After reduction and alkylation, the labeled material was analyzed by reverse-phase HPLC, which has allowed the separation of the two subunit chains of PDGF (3). Only one peak of radioactivity was obtained (Fig. 3), with an elution position exactly corresponding to the position of the PDGF A chain.

Blot-Hybridization Analysis of PDGF A and B Chain mRNA in Melanoma Cell Lines. Blots of poly(A)$^+$ RNA from lines WM 9, SW 691, WM 115, WM 239A, and WM 266-4 were hybridized with ^{32}P-labeled PDGF A and B chain cDNA (Fig. 4). A relative abundance of A chain transcripts sized 1.9, 2.3, and 2.8 kilobases was found in WM 115, WM 239A, and WM 266-4, whereas only WM 115 contained a corresponding proportion of B chain (*SIS*) transcripts.

DISCUSSION

We have demonstrated the production of PDGF-like activity by human melanoma cell lines, the histogenetic origin of which has been proven by their expression of enzymatic and immunological markers (21). Repeated analyses of conditioned medium from cultured normal melanocytes failed to reveal any PDGF receptor-competing activity. Thus, it appears that production of PDGF-like factor may be related to progressive events in melanoma development. However, the production of PDGF-like activity does not appear to be a consistent feature of melanoma-derived cell lines because only three of five cell lines tested revealed any PDGF-like activity. Moreover, the three positive cell lines were all derived from the same patient. In a previous study of three different human melanoma-derived cell lines, none were found to produce *SIS* transcripts (32); whether any of these lines expresses the A chain gene and/or produces PDGF receptor-competing activity has not been determined.

The secreted melanoma-derived PDGF appears as a homogeneous M_r 31,000 species and under reducing conditions as subunits of M_r 17,000 and M_r 16,500. In this respect, the factor is identical to that released by a number of other human cell lines of glioma and sarcoma origin (13, 15). Since the WM 266-4 cells produced only the PDGF A chain, it is not clear

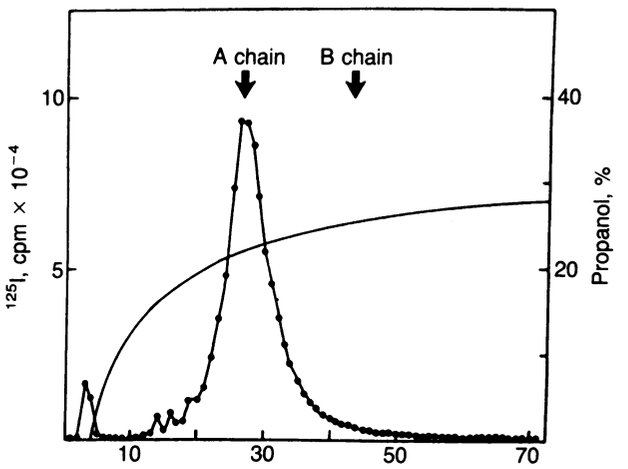

FIG. 3. Elution pattern of reduced and alkylated melanoma-derived, PDGF-like growth factor on reverse-phase HPLC. Purified melanoma-derived factor was labeled with ^{125}I by the method of Bolton and Hunter, subjected to reduction and alkylation, applied to an HPLC RP8 column (Merck), and eluted with a nonlinear gradient of propanol in 1 M acetic acid/2 M guanidine·HCl (6, 28). The elution positions of ^{125}I-labeled PDGF A and B chains, determined by a separate run, are indicated by arrows.

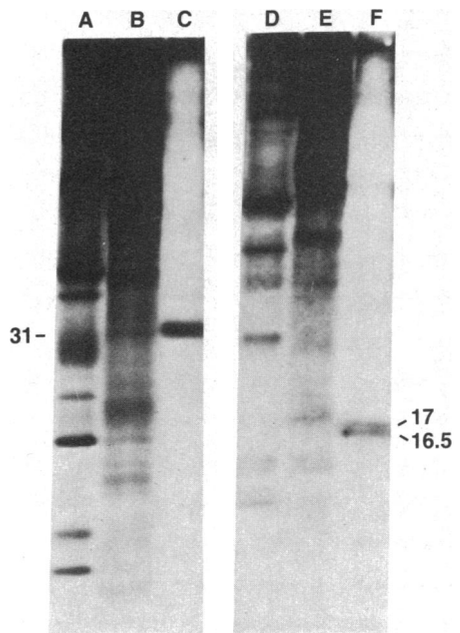

FIG. 2. Immunoprecipitation of the melanoma-derived, PDGF-like growth factor. WM 266-4 cells were metabolically labeled with [^{35}S]cysteine, and culture supernatant was harvested after a 2-hr chase in the absence of isotope. Incubation with control IgG and anti-PDGF IgG, adsorption on protein A-Sepharose, and NaDodSO$_4$/gel electrophoresis under nonreducing or reducing conditions were performed as described. Relative molecular weights are shown × 10^{-3}. Lanes: A and D, medium; B and E, normal rabbit serum; C and F, anti-PDGF antiserum; A–C, nonreduced; D–F, reduced. Exposure was for 2 days at −70°C.

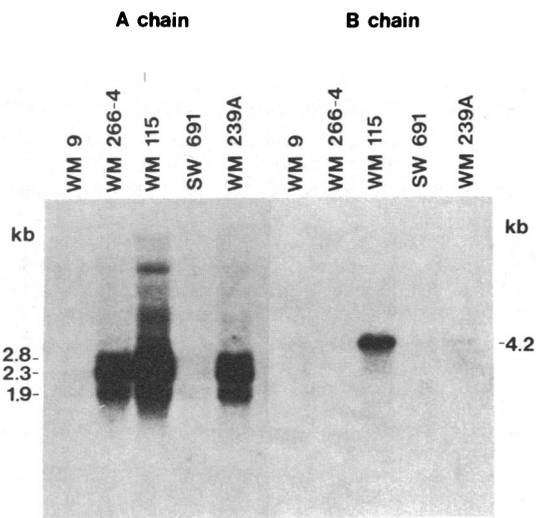

FIG. 4. Blot-hybridization analysis of poly(A)$^+$ RNA isolated from melanoma cells, hybridized to a ^{32}P-labeled PDGF A chain (*Left*) and PDGF B chain (*SIS*) (*Right*) cDNA probes. kb, Kilobases.

why the factor migrates as a doublet after reduction, but this might reflect differences in glycosylation of the subunits; the A chain possesses one possible site for N-linked glycosylation (7, 13). Alternatively, the difference in relative molecular weight might reflect alternative cleavage sites at the C terminus (cf. ref. 13) or alternative splicing of the transcript. Note that the A chain mRNA consistently appears as three discrete species on blot-hybridization analysis (1.9, 2.3, and 2.8 kilobases) (ref. 13 and Fig. 4).

The molecular makeup of human PDGF has not been unequivocally determined. The factor clearly consists of dimers of A and B chains, but it is not known whether they are assembled as homodimers or as a heterodimer. However, the respective gene sequences have evolved from a common ancestral gene with an almost 60% conservation in amino acid sequence of the mature gene products (7, 13). Available data show that the structural homology is matched by functional similarities of the chains when assembled as homodimers. Thus, the mature v-*sis* gene product (33) as well as porcine PDGF (34) exists as dimers of the B chain, whereas the osteosarcoma-derived growth factor (28) and the melanoma-derived product of the present investigation are assembled as A chain homodimers; all of these molecules are mitogenically active, although the relative specific activity of the various forms of the respective factors has not been determined. However, it remains possible that the two subunit chains differ functionally. The B chain gene has a proven transforming potential, both as transduced sequences in simian sarcoma virus and as c-*sis* cDNA (35) or genomic DNA (36) as demonstrated by gene-transfer experiments on NIH 3T3 cells. It remains unclear whether the A chain gene has a similar function and whether its expression in human tumor cells is of significance for their neoplastic character.

All three melanoma cell lines that produced PDGF-like activity expressed a relative abundance of the A chain mRNA, whereas only the cell line derived from the primary tumor expressed a comparable amount of B chain (*SIS*) transcripts. Thus, it appears that the two structural genes encoding human PDGF can be independently expressed, at least in tumor cells; similar findings have been made recently on human cell lines derived from sarcomas and gliomas (13). The presence of identical chromosome markers in the WM 115 tumor and its metastases indicates a monoclonal origin of the tumor (21). Whether the loss of *SIS* expression is related to tumor progression in human melanoma or is a fortuitous phenomenon remains to be established. However, human tumor cell lines express the A chain more frequently than the B chain (ref. 13), an observation consistent with the notion that expression of *SIS* may be lost after long-term growth of the tumor cells, whereas expression of the A chain gene persists. Since melanocyte-derived cells of all stages of tumor development can now be analyzed in cell culture, we believe that this system lends itself to the study of the possible relationship of tumor progression and PDGF synthesis.

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