Abstract

Platelet-derived growth factor (PDGF) has been directly implicated in developmental and physiological processes, as well as in human cancer and other proliferative disorders. We have recently isolated and characterized a novel protease-activated member of the PDGF family, PDGF D. PDGF D has been shown to be proliferative for cells of mesenchymal origin, signaling through PDGF receptors. Comprehensive and systematic PDGF D transcript analysis revealed expression in many cell lines derived from ovarian, renal, and lung cancers, as well as from astrocytomas and medulloblastomas. β PDGF receptor profiling further suggested autocrine signaling in several brain tumor cell lines. PDGF D transforming ability and tumor formation in SCID mice was further demonstrated. Exploiting a sensitive PDGF D sandwich ELISA using fully human monoclonal antibodies, PDGF D was detected at elevated levels in the sera of ovarian, renal, lung, and brain cancer patients. Immunohistochemical analysis confirmed PDGF D localization to ovarian and lung tumor tissues. Together, these data demonstrate that PDGF D plays a role in certain human cancers.

Introduction

To identify pharmaceutical targets for development of protein therapeutic drugs, mAb, and small molecule antagonists, we are searching human databases that contain coding regions for virtually all expressed genes in a specific tissue, including rarely expressed and underrepresented genes that are relevant to human disease, we identified the protease-activated PDGF2 D (1, 2).

Although PDGFs play an important role in normal development, accumulating evidence suggests that their abnormal expression also contributes to a variety of diseases. This is emphasized by the fact that PDGFs and their receptors are currently under investigation as targets in numerous proliferative disorders, including cancer and cardiovascular and fibrotic diseases (3, 4). The PDGF family currently consists of at least four distinct genes, PDGF A, PDGF B, PDGF C, and PDGF D, whose gene products selectively signal through two distinct PDGFRs to regulate cellular functions. PDGF A and B transforming ability and tumor formation in mouse xenograft models have been thoroughly characterized (3–6). Recently, however, database mining has resulted in the discovery of PDGF C (7, 8) and PDGF D (1, 2). PDGF A and PDGF C were reported to selectively activate α PDGFR (7, 9).

In a subsequent report, PDGF C was shown to require an α PDGFR-related mechanism to activate the β PDGFR (10). PDGF B and PDGF D have been shown to activate both α and β PDGFRs (1, 2).

Here, we investigate the role of PDGF D in neoplasia. We first demonstrate that many cancer cell lines express PDGF D mRNA and that some of these cell lines also express β PDGFR, suggesting autocrine signaling events. Consistent with its previously described growth stimulatory properties, we show that PDGF D induces β PDGFR autophosphorylation, transforms NIH 3T3 fibroblasts, and promotes tumor formation in vivo. Furthermore, PDGF D is detected in many human cancer sera and tumor tissues. Collectively, these observations suggest that PDGF D plays a role in some human malignancies.

Materials and Methods

Cells. Mammalian tumor-derived cell lines (American Type Culture Collection), 293-EBNA cells (Invitrogen), and endothelial cells (Clonetics) were obtained from commercial sources. Human PDGF A and PDGF B were purchased from R&D Systems.

RTQ-PCR Expression Analysis. RNA samples derived from normal human tissues were obtained commercially (Clontech; Invitrogen; Research Genetics). Cell lines were grown according to specifications. RNAs were harvested, and PCR was performed as described previously (1) using TaqMan reagents (PE Applied Biosystems). RNAs were normalized using human β-actin and glyceraldehyde-3-phosphate dehydrogenase TaqMan probes according to the manufacturer’s instructions. Equal quantities of normalized RNA were used as templates in PCR reactions with PDGF D-specific reagents to obtain C(T) values. For graphic representation, C(T) values were converted to percentage expression, relative to the sample exhibiting the highest level of expression. Primers used for PDGF D analysis were: forward primer (5'-CGCTTGGGATCATCATATTGAG-3'); reverse primer (5'-CGGTATCGAG-GCACGTACATAC-3'); TaqMan probe (5'-FAM-TCCAGGTCACACTTACACTGTTCCCGCTCA-TAMRA-3'). Primers used for PDGF B analysis were: forward primer (5'-AAGATCGAGATTGTGCGGAAGA-3'); reverse primer (5'-ACTTGCCATGGCAGGTGGTCT-3'); TaqMan probe (5'-FAMCCAGGTCACCGTGCCATTAAT-TAMRA-3').

β PDGFR Phosphotyrosine Analysis. Cell lysates were prepared from 3 × 10^6 T98G or SK-N-AS cells, as well as 1 × 10^6 NIH 3T3-PDGF D transfectants starved 24 h and immunoprecipitated with control or β PDGFR antibody as described previously (1). Filters were probed with anti-PY20 (Santa Cruz Biotechnology, Inc.) or β PDGFR antibody (Santa Cruz Biotechnology, Inc.), and bands were visualized by enhanced chemiluminescence (Amersham).

NIH 3T3 Transformation and SCID Mouse Tumor Assay. NIH 3T3 cells were transfected with pMT (11)-PDGF D using Lipofectamine-Plus according to the manufacturer’s protocol (Life Technologies, Inc., Bethesda, MD); pMT-PDGF D was engineered to express the proteolytically processed and thus activated PDGF D p35 as described previously (1). NIH 3T3 cells were supplemented with 10% CS (Life Technologies, Inc.) 5 h post-transfection. Two days after transfection, PDGF D-transfected cells were split into DMEM/10% CS supplemented with 600 μg/ml geneticin (Life Technologies, Inc.)

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2 The abbreviations used are: PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; RTQ, real-time quantitative; C(T), threshold cycle; CS, calf serum; mAb, monoclonal antibody; HRP, horseradish peroxidase; NCI, National Cancer Institute; CNS, central nervous system.
Inc.). To generate control cells, NIH 3T3 cells were transfected with control pMT vector and selected as described above. After 2 weeks of culture, pools of transfected cells were trypsinized, neutralized with DMEM/10% CS, washed with PBS, and counted. Transfected cells in PBS (2 × 10⁶) were injected into the lateral subcutis of female SCID mice. Tumors were measured with calipers every 3–4 days.

Generation of Fully Human PDGF D Monoclonal Antibodies. Fully human PDGF D mAbs were generated as described previously (12) with the following modifications. Briefly, the human IgG2-bearing XenoMouse strain (8–10 weeks old) was immunized twice weekly by footpad injection with 10 μg of V5-tagged soluble PDGF D (1) in complete Freund’s adjuvant (12). Hybridomas were generated using electro-cell fusion. mAbs did not recognize PDGF A, PDGF B, or PDGF C by ELISA or immunoprecipitation.

PDGF D ELISA. A sandwich ELISA was developed to quantify PDGF D levels in human serum. The two fully human mAbs (1.6 and 1.17) used in the sandwich ELISA recognized different epitopes on the PDGF D molecule (data not shown). The ELISA was performed as follows: 50 μl of capture antibody (mAb 1.6) in coating buffer [0.1 M NaHCO₃ (pH 9.6)] at a concentration of 2 μg/ml was coated on ELISA plates (Fisher). After incubation at 4°C overnight, the plates were treated with 200 μl of blocking buffer (0.5% BSA, 0.1% Tween 20, 0.01% Thimerosal in PBS) for 1 h at 25°C. The plates were washed (three times) using 0.05% Tween 20 in PBS (washing buffer). Normal or patient sera (Clinomics; Bioreclamation; Cooperative Human Tissue Network) were diluted in blocking buffer containing 50% human serum. The plates were incubated with serum samples overnight at 4°C, washed with washing buffer, incubated with serum samples overnight at 4°C, washed thoroughly with PBS. After incubation with streptavidin-HRP conjugate for 10 min, a solution of diaminobenzidine was applied onto the sections to visualize the immunoreactivity. For the isotype control, sections were incubated with biotinylated isotype-matched negative control mAb at 5 μg/ml for 30 min at 25°C instead of biotinylated PDGF D mAb.

Results and Discussion

PDGF D Transcript Expression and Signaling in Human Cancer Cell Lines. We examined the mRNA expression profile of PDGF D in cell lines derived from cancers of multiple origins using RTQ-PCR. The primer/probe set used was designed to be PDGF D specific and, as such, did not detect other known PDGF family members. In a representative experiment (Fig. 1A), PDGF D was most highly expressed in the lung cancer cell lines, such as NCI-H596, SW900,
HOP62, and A549. Moderate levels of PDGF D were found in ovarian cancer cell lines, such as IGROV-1 and OVCAR-8. Several CNS-derived astrocytoma/glioblastoma and neuroblastoma cell lines, such as U251, SNB-19, SNB-75, SK-N-AS, and SW1783, also showed moderate expression. PDGF D was also expressed to a lesser extent in most melanoma cell lines tested. In a separate set of experiments (Fig. 1B), PDGF D was highly expressed in the lung cancer cell line NCI-UMC-11, as well as the astrocytoma-derived SF-295 and neuroblastoma-derived T98G. An ovarian (RL95-2), renal (Caki-2), lung (NCI-H146), and astrocytoma (SK-N-SH) cell line showed moderate expression. Of note, PDGF D was detected in only 2 of 17 colon cancer cell lines tested. These data demonstrate that PDGF D transcript is expressed in many ovarian, lung, renal, and brain cancer-derived cell lines.

To gain further insight into PDGF D function (1), we examined mRNA expression of PDGF B, PDGF D, and the PDGFR α and β PDGFRs through which they signal. The expression analysis was performed using RNA derived from a set of CNS-derived tumor cell lines. Data summarized in Fig. 1C demonstrated that PDGF D and PDGF B transcript were expressed in 10 and 7 of the 17 cell lines, respectively. Five cell lines expressed mRNA for both PDGF D and PDGF B. Five cell lines had only PDGF D mRNA and 2 expressed only that of PDGF B, whereas 5 cell lines contained neither transcript.

Autocrine signaling was suggested for 6 of 11 astrocytoma/glioblastoma cell lines and 2 of 4 medulloblastoma/medulloblastoma cell lines based on coexpression of PDGF D mRNA with the α and β PDGFR transcripts (Fig. 1C). PDGF D was coexpressed with the β PDGFR alone in 2 medulloblastoma/medulloblastoma cell lines. PDGF B was expressed with the β PDGFR in all 7 PDGF B-positive lines. PDGF D and PDGF B were coexpressed in 5 of these lines. We further found that PDGF D was not coexpressed with the β PDGFR in the vast majority of other cancer cell lines tested with the exception of OVCAR-8, G401, and HOP62 (data not shown).

To test if PDGF D could participate in a functional autocrine loop, we examined β PDGFR autophosphorylation in T98G and SK-N-AS cells that possess little or no PDGF B transcript expression. As shown in Fig. 1D, β PDGFR tyrosine phosphorylation was detected in antiphosphotyrosine immunoblots of T98G cell lysates first immunoprecipitated with a β PDGFR-specific antibody. β PDGFR tyrosine phosphorylation, albeit to a lesser extent, was also observed in SK-N-AS immunoprecipitates. Furthermore, potent β PDGFR tyrosine phosphorylation was detected in NIH 3T3-PDGF D transfectants (Fig. 1D). Tyrosine phosphorylated β PDGFR was not detected using control antibody (Fig. 1D) or control NIH 3T3 transfectant immunoprecipitates (data not shown). These results show that although PDGF D is expressed as a paracrine factor by many cancer cell lines, an autocrine signaling component exists in some astrocytoma and medulloblastoma cell lines.

**PDGF D Induces Morphological Transformation In Vitro and Tumor Formation In Vivo.** To determine whether ectopic PDGF D expression induced cell transformation, NIH 3T3 transfectants were generated. The resulting NIH 3T3 PDGF D transfectants exhibited foci of morphologically transformed cells characterized by a dense, disorganized pattern of growth, comprised of individual cells found to be spindly in shape with increased refractivity. NIH 3T3 cells transfected with control vector retained a normal morphology (Fig. 2, A...
and B). In separate experiments, NIH 3T3 cells treated in culture with purified PDGF D for 2 days also became morphologically transformed in appearance, whereas mock-treated cells remained unchanged (data not shown).

To assess the ability of PDGF D to induce tumor formation in vivo, PDGF D and control vector transfectants were injected s.c. into SCID mice. By 18 days, all of the animals injected with PDGF D-transfected cells possessed rapidly growing tumors, whereas none of the animals injected with control cells had tumors (Fig. 2C). After 25 days, the tumorigenicity of PDGF D was evident with a mean tumor size approaching 2500 mm$^3$. Examination of lung and other tissues detected no metastases. All of the data clearly demonstrate that PDGF D has potent growth-stimulating potential in vitro and oncogenic potential in vivo.

**PDGF D Serum Levels in Patients with Cancer.** As an additional step in determining whether PDGF D might be involved in human cancer, we surveyed serum levels from patients with various types of malignancy. Serum PDGF D concentrations were assessed using a quantitative sandwich ELISA with two fully human mAbs raised against PDGF D. The ELISA was specific for PDGF D and had a sensitivity of 4 ng/ml. As shown in Fig. 3, PDGF D was expressed at concentrations $>10$ ng/ml in 28% of sera from patients with cancer ($n = 245$), compared with 6% of normal sera ($n = 50$). The mean PDGF D serum concentration was significantly elevated in sera from patients with medulloblastoma ($P = 0.004$) and astrocytoma ($P = 0.019$), as well as ovarian ($P = 0.001$), lung ($P = 0.004$), bladder ($P = 0.001$), renal ($P = 0.002$) and breast ($P = 0.002$) cancers. In some ovarian cancer and medulloblastoma patients, concentrations $>40$ ng/ml were detected. The growth factor was below detection levels in sera from patients with lymphoma and melanoma. The mean serum levels of PDGF D in cancer patients ranged from 4 to 15 ng/ml, compared with a concentration of $<4$ ng/ml in normal individuals. These data demonstrate that PDGF D is elevated in the sera of patients with certain malignancies.

**Immunohistochemical Analysis of PDGF D in Cancer.** The elevated level of PDGF D in certain cancer cell lines and sera suggested that PDGF D might be expressed in human tumor tissue. Therefore, the expression of PDGF D was examined in human cancers by immunohistochemical analysis. The PDGF D mAb used in this study specifically recognized PDGF D and not PDGF A, B, or C based on either ELISA or Western blot analysis (data not shown). As shown in Fig. 4B, normal lung tissue did not stain for PDGF D. However, immunohistochemical analysis of PDGF D in lung tumor tissue detected PDGF D staining (Fig. 4D). The PDGF D mAb showed uniform staining of normal ovary (Fig. 4F). Staining of ovarian tumor tissue revealed increased PDGF D staining of tumor cells and surrounding stroma (Fig. 4H). Ovary and lung tissues, both normal and tumor, were uniformly negative when stained with the control mAb. In situ hybridization experiments will be necessary to determine the precise cellular origin of the detected PDGF D. All of these data suggest that PDGF D is aberrantly localized in some specific tumor tissues and may play a role in tumorigenesis.

The role of autocrine PDGF A and PDGF B signaling in cancer has been well documented (3–6). Here we found that the PDGF D transcript is expressed in an autocrine signaling loop with the $\beta$ PDGFR in several astrocytoma/glioblastoma and medulloblastoma/neuroblastoma cell lines. Interestingly, the astrocytoma cell lines all expressed PDGF A (except SK-N-AS), PDGF C, and $\alpha$ PDGFR transcript. PDGF B mRNA was expressed in some of the astrocytoma and medulloblastoma cell lines as well. Therefore, in cell lines that contain both PDGFRs, PDGF B, PDGF C, and PDGF D would be expected to complete autocrine signaling events through $\alpha$ and $\beta$ PDGFRs. Although the breadth of PDGF and PDGFR expression underscores a role for autocrine PDGF signaling in brain tumor formation, the contribution of each PDGF and PDGFR to these events will be the subject of future investigations.

We reported previously that PDGF D proliferative activity depends on the proteolytic removal of its CUB domain and that the p84 uncleaved ligand did not interact with PDGFRs (1). Consistent with these observations, we found that only the p35 active form of PDGF D caused transformation of NIH 3T3 cells and tumor formation in mice (Fig. 2). Thus, PDGF D transformation and tumorigenesis also depended on the proteolytic removal of the CUB domain. Because CUB domains have also been shown to specify protein-protein interactions (13), it is tempting to speculate that the PDGF D CUB domain may play the dual role of specifying protease interaction, as well as blocking receptor interaction. Although the PDGF D-activating protease(s) have yet to be identified, enzymes such as stromelysin and MMP-3, are induced after PDGFR activation and also in many cancers (14). The role of these proteases in PDGF D activation and human cancer progression is currently under investigation.

PDGF D maps to a human chromosomal locus (11q23-24) of recognized genomic instability (15). Coincidentally, this region also

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Figure 3. Detection of PDGF D in human normal and cancer sera. PDGF D in human serum was quantified using a sandwich ELISA as described in “Materials and Methods.” The sandwich ELISA did not recognize PDGF A, PDGF B, or PDGF C. The PDGF D concentration for an individual clinical serum sample. PDGF D serum concentrations are grouped according to the patient disease indication. The number ($n$) of patients for a given clinical indication is provided, along with the mean PDGF D concentration in ng/ml.
encodes matrix metalloproteinases (16) and shows gene copy number variations in some diseases, e.g., Jacobsen’s syndrome is marked by craniofacial, heart, and glandular abnormalities, as well as lack of brain development (17). These disease manifestations might be explained in part by aberrant growth factor expression. Of particular interest is the amplification about this locus in glioblastoma multiforme (18) and childhood medulloblastoma (19). Amplifications or deletions in the region of chromosome 11q23-24 have also been implicated in lung cancer (20), ovarian cancer (21), and primary sarcomas (22). Of note, lung A549 cells that show elevated PDGF D transcript also possess 11q23-24 amplification (20). Considering the role that PDGFs play in malignancy (3), it is possible that inappropriate expression of PDGF D, whether paracrine or autocrine, may contribute to cancers associated with chromosome 11q23-24 abnormalities. The elevated PDGF D expression that we observe in human cell lines, sera, and cancer tissues supports such a hypothesis.

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References


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