The Dermatofibrosarcoma Protuberans-associated Collagen Type Iα1/Platelet-derived Growth Factor (PDGF) B-Chain Fusion Gene Generates a Transforming Protein That Is Processed to Functional PDGF-BB

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ABSTRACT

Dermatofibrosarcoma protuberans (DFSP) displays chromosomal rearrangements involving chromosome 17 and 22, which fuse the collagen type Iα1 (COL1A1) gene to the platelet-derived growth factor (PDGF) B-chain (PDGFB) gene. To characterize the functional and structural properties of the COL1A1/PDGFB fusion protein, we generated a stable NIH3T3 cell line that contained a tumor-derived chimeric gene resulting from a COL1A1 intron 7-PDGFB intron 1 fusion. Expression of the fusion protein led to morphological transformation and increased growth rate of these cells. The PDGF receptor kinase inhibitor CGP57148B reversed the transformed phenotype and reduced the growth rate of COL1A1/PDGFB expressing cells but had no effects on control cells. The presence of dimeric COL1A1/PDGFB precursors was demonstrated through PDGFB immunoprecipitations followed by immunoblotting with COL1A1 antibodies. Pulse-chase studies demonstrated that the COL1A1/PDGFB precursor was processed to an end product that was indistinguishable from wild-type PDGF-BB. Finally, COL1A1/PDGFB-expressing cells generated tumors after s.c. injection into nude mice, and tumor growth was reduced by treatment with CGP57148B. We conclude that the COL1A1/PDGFB fusion gene associated with DFSP contributes to tumor development through ectopic production of PDGF-BB and the formation of an autocrine loop. Our findings, thus, suggest that PDGFB receptors could be a target for pharmacological treatment of DFSP and giant cell fibroblastoma, e.g., through the use of PDGF receptor kinase inhibitors such as CGP57148B.

INTRODUCTION

DFSP and its juvenile form, GCF, are highly recurrent, infiltrative skin tumors of intermediate malignancy (1). They are occasionally misdiagnosed as benign lesions such as dermatofibroma and neurofibroma, leading to improper primary management. The current treatment of DFSP/GCF is entirely surgical, and recurrence rates of up to 50% following removal have been reported (2). The histogenesis of DFSP/GCF is still controversial; however, the strongest evidence supports a fibroblastic origin (2).

All DFSP and GCF cases studied to date have been found to contain a fusion of COL1A1 to PDGFB, as a result of rearrangements involving chromosomes 17 and 22 (3, 4). The COL1A1 gene, located on 17q22, encodes the major component of type I collagen, the most abundant protein in the body, which is produced primarily by fibroblasts. PDGFB (or c-sis proto-oncogene), located on 22q13, is the cellular equivalent of the v-sis oncogene, which causes simian sarcoma (5, 6). PDGFB-BB, the homodimer formed by disulfide linking, is a potent growth factor that acts as a mitogen and chemotactic for a variety of connective tissue cells (7). It exerts its action through two structurally similar tyrosine kinase receptors, the PDGF α- and β-receptors, on the surface of target cells (8). The coexpression of PDGFB and its receptors has been described in several tumors, including DFSP, and involvement of a PDGFB autocrine loop in tumorigenesis has been suggested (9). The PDGF β-receptor pathway has also been implicated in chronic myelomonocytic leukemia, in which a translocation gives rise to a ligand-independent constitutively active form of the PDGFB β-receptor (10, 11).

The transforming potential of the COL1A1/PDGFB fusion gene was recently demonstrated (12). In this study, we have generated a NIH3T3 cell line that expresses a tumor-derived chimeric gene. The cell line has been used to characterize the fusion protein functionally and structurally.

MATERIALS AND METHODS

COL1A1/PDGFB Cosmid Isolation and Establishment of NIH3T3 Cell Lines. The COL1A1/PDGFB fusion transcript and genomic breakpoint of TNM2, a DFSP recurrence, has been characterized previously, and a SuperCos1 cosmid library has been constructed (4). This library was screened using previously described probes: a COL1A1 probe, 5′ COL, and a PDGFB probe, pex7 (13). The following primers were used in PCR amplification and sequencing: fd.f2, 5′-CCG AGG AGT AAG TGG; SuperCos1, 5′-GCA ATT AAC CCT CAC TAA AG; SuperCos2, 5′-CCG CAT AAT ACG ACT CAC TAT; and clai.int1, pdgfb.int, clai.ex1t, CX1.TS1, CX1.TS3, CX1.TS4, CX1.TS5, FF1.tr, and PDGFB-6 (4).

NIH3T3 cells (ATCC CRL-1658) were maintained in DMEM supplemented with 10% calf serum using standard conditions. Two μg of cosmDNA were transfected into 3 × 10⁵ NIH3T3 cells plated in 35 × 10⁶ mm plates in serum-free DMEM using Lipofectamine, according to the recommendations of the supplier (Life Technologies, Inc., Gaithersburg, MD). Transfectants were selected in 600 μg/ml genetin (Life Technologies, Inc.) before passaging.

Genomic DNA was extracted from cells using standard protease K digestion followed by phenol/chloroform extraction. The presence of the fusion gene was confirmed by PCR of genomic DNA using the primers fd.f2 and pdgfb.int (see above). Total RNA was extracted from cells using acidic guanidinium thiocyanate-phenol-chloroform extraction (14). Reverse transcriptase-PCR and sequencing was performed as described (4). The cell lines B5/5 (COL1A1/PDGFB transfected) and CNEG-1 (negative control) were used in this study.

Drugs and Immunological Reagents, in Vitro Growth Characterization, Metabolic Labeling, Immunoprecipitation, and Immunoblotting. Brefeldin A (Sigma Chemical Co., St. Louis, MO) was prepared as a stock solution of 6 mg in ethanol. The PDGF receptor tyrosine kinase inhibitor CGP57148B (15) was prepared as a 100 μM stock solution in PBS. Goat anti-PDGF β-receptor antibody P-20 (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal antiphosphotyrosine antibody PY20 (Transduction Laboratories, Lexington, KY), and rat antihuman pro-collagen I NH₂-terminal monoclonal

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Abbreviations used are: DFSP, dermatofibrosarcoma protuberans; GCF, giant cell fibroblastoma; COL1A1, collagen type Iα1; PDGFB, platelet-derived growth factor, PDGFB, PDGFB B-chain.
antibody (Chemicon, Tunicula, CA) were used as recommended by the suppliers.

For studies of the in vitro growth rates, cells were plated in six-well plates (5 × 10^4 cells/well) in DMEM supplemented with 10% FCS in the presence or absence of 1.0 µM CGP57148B. Medium exchange was performed three times per week. After trypsinization, cells were counted using a Coulter particle counter.

Metabolic labeling was performed as follows: cells were labeled for 2 h with 0.1 µCi/ml [35S]cysteine in cysteine-free MCDB 104 medium supplemented with 1 mg/ml BSA. In pulse-chase experiments, chase was performed in medium containing 250 µg/ml unlabeled cysteine. Immunoprecipitations were performed using a PDGF-BB antiserum (16), as described previously (17).

To detect the COLIA1/PDGFB fusion protein through COLIA1 immunoblotting, we incubated subconfluent CNEG-1 and B5/5 cells with 10 µM breflidin A for 4 h at 37°C. Cell lysates were prepared and immunoprecipitated with PDGFB-BB antiserum, as described above. After 7% SDS-PAGE and semidry transfer to nitrocellulose membranes, membranes were incubated with rat antihuman pro-collagen I antibody. After incubation with horseradish peroxidase-conjugated antirat antibody (Amersham) followed by ECL.

For immunoprecipitation and immunoblotting of PDGF β-receptors, cells were incubated overnight in the absence or presence of CGP57148B in serum-free DMEM containing 1 mg/ml BSA, washed with PBS, and lysed by 15 min of incubation in 1% NP40, 0.15 M NaCl, 20 mM Tris (pH 7.5), 5 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylool, and 1 mM orthovanadate at 4°C. After centrifugation, lysates were incubated with PDGF β-receptor antiserum PDGFR-3 (18) for 2 h at 4°C. After collection of immune complexes with protein A-Sepharose, complexes were washed three times with lysis buffer and once with 20 mM Tris-HCl (pH 7.5); immune complexes were then eluted and subjected to 7% SDS-PAGE. Proteins were transferred to nitrocellulose membranes using semidydro electrophoresis. Membranes were blocked and probed with phosphotyrosine antibody PY20, and after incubation with horseradish peroxidase-conjugated antitumor antibodies, tyrosine-phosphorylated proteins were detected by enhanced chemiluminescence (ECL; Amersham). For detection of PDGF β-receptor expression, membranes were stripped and incubated with PDGFB receptor antiserum P-20. PDGF receptors were visualized by incubation with horseradish peroxidase-conjugated antirabbit antibodies (Amersham) followed by ECL.

**Tumorigenicity Assays and Histological Characterization of s.c. Tumors.** B5/5 and CNEG-1 cells (2 × 10⁶ in 50 µl of PBS) were injected s.c. into the left flank of 6–10-week-old male BALB/c nu/nu mice; 10 mice were injected with each cell type. Half of the mice in each group were given 50 mg/kg × kg⁻¹ × day⁻¹ CGP57148B in 200 µl of PBS, and the other half were given 200 µl of PBS only. Treatment was started on the day following injection of tumor cells. Drug was administered p.o. by tube feeding once daily. Animals were individually caged and fed animal chow ad libitum. Tumor diameter was measured every 3 days, and tumor volume was subsequently calculated using the formula V = π × 6₁⁻¹ × a² × b, where a and b represent the shorter and the longer diameters of the tumor, respectively. For histological analysis, tumors were fixed in 4% paraformaldehyde and embedded in paraffin, and 5-µm sections were subjected to H&E and Picro-Ponceau stainings.

**RESULTS**

Isolation of a COLIA1/PDGFB Cosmid and Establishment of COLIA1/PDGFB-expressing NIH3T3 Cell Lines. TNM2, a DFSP case displaying a ring chromosome, contains a COLIA1/PDGFB chimeric gene with a COLIA1 intron 7-PDGFB intron 1 fusion. From a SuperCos1 cosmid library of tumor TMN2, 15 positive clones were isolated and characterized by end-sequencing. One cosmid, CC12, was found to contain the full chimeric gene. The position of the 5' and 3' ends of the fusion gene was extrapolated from end-sequencing,primer walking, and insert size estimation (35 kb). The 5' end of the chimeric gene was located in the middle of the cosmid, and the 3' end was located 1 kb from the end of the cosmid. A negative control cosmid, CNEG, was randomly picked from the TNM2 cosmid library and confirmed by PCR to contain neither COLIA1 nor PDGFB segments. Cosmids CC12 and CNEG were transfected into NIH3T3 cells using Lipofectamine and, after G418 selection, clones of resistant cells were obtained. CC12-transfected clones were confirmed by PCR to contain the COLIA1/PDGFB fusion gene. Reverse transcriptase-PCR analysis revealed that these cells produced the expected chimeric transcript with a COLIA1 exon 7-PDGFB exon 2 fusion. This was confirmed by Northern blot analysis, which detected a chimeric 3.4-kb transcript (data not shown). One COLIA1/PDGFB-positive clone, B5/5, was chosen for further analysis together with CNEG-1, a clone derived from the transfection with the CNEG cosmid.

**Expression of COLIA1/PDGFB Protein Leads to Transformation of NIH3T3 Cells through Activation of PDGF β-Receptors.** To investigate the potential transforming ability of the COLIA1/PDGFB protein, we compared the morphology and growth pattern of B5/5 and CNEG-1 cells. As shown in Fig. 1A, B5/5 cells grew in a disorganized manner and displayed a spindle-shaped morphology that is typical of transformed cells, with loss of contact inhibition. Addition of the PDGF receptor kinase inhibitor CGP57148B dramatically altered the morphology of B5/5 cells to a phenotype that is indistinguishable from that of the control cells, indicating that the altered morphology occurred as a consequence of PDGF receptor activation.

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**Fig. 1.** COLIA1/PDGFB expression leads to in vitro transformation through activation of PDGF receptors. A, CNEG-1 (control) and B5/5 (COLIA1/PDGFB) cells were cultured in the absence or presence of 1.0 µM CGP57148B for 6 days in DMEM containing 10% FCS. Microphotographs were taken with a phase contrast microscope. B, CNEG-1 cells and B5/5 cells were plated in six-well plates at 2.5 × 10⁴ cells/well in DMEM containing 10% FCS and grown in the absence (□) or presence (●) of 1.0 µM CGP57148B. At indicated times, cells were trypsinized, and cell number was determined with a Coulter counter. C, CNEG-1 (control) and B5/5 (COLIA1/PDGFB) cells were grown in the absence or presence of CGP57148B, lysed, and subjected to PDGF β-receptor immunoprecipitation and SDS-gel electrophoresis. After transfer to membranes immunoblotting with phosphotyrosine antibodies (top) and PDGF β-receptor antiserum (bottom) was performed.

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(Fig. 1A). The effects of CGP57148B on the growth rate of B5/5 and CNEG-1 cells were also analyzed (Fig. 1B). Whereas the growth rate of CNEG-1 cells was unaffected by CGP57148B, the growth of B5/5 cells was dramatically reduced in the presence of the PDGF receptor kinase inhibitor. The difference in growth rate between CGP57148B-treated CNEG-1 and B5/5 cells most likely reflect clonal variations between the two NIH3T3 cell line derivatives. However, the clear difference between the two cell lines with regard to CGP57148B sensitivity strongly indicate that autocrine PDGF receptor signaling contributes to the growth of B5/5 cells but not to the growth of CNEG-1 cells.

To directly demonstrate the presence of activated PDGF β-receptors in B5/5 cells, receptors were isolated, from cells grown in the absence or presence of CGP57148B, by immunoprecipitations and were subjected to phosphotyrosine immunoblotting (Fig. 1C). Upon immunoblotting, B5/5 extracts display a strong signal, indicating autocrine stimulation of PDGF-β-receptors by the COLIA1/PDGFB-encoded protein. Furthermore, the steady-state level of PDGF β-receptors in B5/5 cells was reduced compared to CNEG-1 cells, consistent with a ligand-induced receptor down-regulation. Finally, the inhibitory effect of CGP57148B on PDGF receptor activation was clearly demonstrated by the finding of dramatically reduced tyrosine phosphorylation of PDGF receptors after treatment (Fig. 1C, Lanes 2 and 4). Studies using mutant forms of PDGF β-receptors have demonstrated that kinase-inactive receptors still display down-regulation upon ligand binding (19). Similarly, it is likely that the reduced receptor levels in CGP57148B-treated B5/5 cells also occur as a consequence of ligand-induced internalization, despite the absence of tyrosine kinase activation.

The COLIA1/PDGFB Fusion Protein Forms a Disulfide-linked Precursor Dimer That Is Processed to Mature PDGF-BB. To characterize the protein(s) encoded by the COLIA1/PDGFB fusion gene, we metabolically labeled the B5/5 cells with [35S]cysteine, and cell lysates were subjected to immunoprecipitations using a PDGF-BB antiserum (Fig. 2A). To enrich for precursor forms, we performed the labeling in the presence of brefeldin A, a drug that blocks ER to Golgi transport and that enriches for PDGF precursor forms (20). For comparison, PDGFB-expressing NIH3T3 cells (21) were included in the analysis. An unreduced M, 110,000 component, which was converted to a M, 60,000 component after reduction, was identified in the lysates of B5/5 cells (Fig. 2A, Lanes 4 and 8). These findings indicate that the COLIA1/PDGFB fusion protein, like PDGFB, forms a dimeric precursor. To strengthen the notion that the M, 110,000 protein observed in B5/5 cells represents a COLIA1/PDGFB fusion protein, we subjected lysates of B5/5 cells to PDGF immunoprecipitations followed by immunoblotting with a collagen antibody. A M, 110,000 component was identified in lysates of brefeldin A-treated B5/5 cells but not in CNEG-1 cells (Fig. 2B).

To study the processing of the COLIA1/PDGFB fusion protein in more detail, we performed pulse-chase experiments using COLIA1/PDGFB- and PDGFB-expressing cells. Analysis of PDGFB-expressing cells revealed a gradual decrease in the cell-associated unprocessed M, 56,000 precursor, with an accompanying increase in cell-associated M, 40,000 and M, 24,000 forms over time (Fig. 3, Lanes 6–8), which is consistent with previous reports (20, 22). Similarly, in the case of B5/5 cells, processed forms of M, 40,000 and M, 24,000 were also found to accumulate with time. In both cell types, very small fractions of the processed forms were secreted into the medium (Fig. 3, Lanes 4, 5, 9, and 10). The distribution between cell-associated and secreted forms of the M, 40,000 species did not vary in a consistent way between COLIA1/PDGFB- and PDGFB-expressing cells (data not shown).

The COLIA1/PDGFB precursor, thus, forms a COLIA1/PDGFB dimeric precursor that is processed to mature forms indistinguishable from wild-type PDGF-BB, both with regard to structure and compartmentalization.

In Vivo Tumorigenesis by COLIA1/PDGFB-expressing NIH3T3 Cells. To investigate the tumorigenic potential of the COLIA1/PDGFB protein, we injected B5/5 cells and CNEG-1 cells into nude mice and monitored them for the appearance of tumor growth. B5/5 cells, in contrast to CNEG-1 cells, formed tumors of measurable size within 2 weeks (Fig. 4). The PDGF receptor kinase inhibitor CGP57148B significantly reduced the growth of B5/5 derived tumors, indicating that an autocrine PDGF receptor stimulation contributed to
DFSP and GCF display chromosomal rearrangements involving chromosome 17q22 and 22q13 (23). These rearrangements fuse the COL1A1 gene (17q22) to the PDGFB gene (22q13; 3, 4). There is wide variation in the position of the fusion position in COL1A1, but it always occurs within the region encoding the α-helical coding domain. The exons of COL1A1 in this region consistently end at the last base of a codon. The breakpoint in PDGFB is always in intron 1. The resulting COL1A1/PDGFB fusion is, therefore, in-frame, because exon 2 of PDGFB starts at the first base of codon 22. This fusion gene thus encodes a protein consisting of a COL1A1 NH2-terminal peptide fused to residues 22–241 of the PDGFB propeptide. The DFSP case TNM2 used in this study displays a ring chromosome, which contains a chimeric gene, the result of a COL1A1 intron 7–PDGFB intron 1 fusion (4). This fusion gene was sufficiently compact to allow cloning in a cosmid vector. The cosmid CC12 was subsequently found to contain the entire chimeric gene, including promoter and regulatory regions. Although the DFSP/GCF histogenesis is still controversial, evidence from both electron microscopy and the involvement of COL1A1, suggest that DFSP/GCF is of fibroblastic origin (2). Because DFSP cells, fibroblasts, and NIH3T3 have all been shown previously to produce collagen and express PDGF β-receptors, we chose NIH3T3 cells as a model for this study (24–27).

To study the functional and structural properties of the COL1A1/PDGFB protein, we stably transfected NIH3T3 cells with CC12 and established a number of cell lines, from which one, B5/5, was selected. The fusion gene transformed NIH3T3 cells into spindle-shaped cells, with a more disorganized growth pattern and the ability to form tumors in nude mice (Figs. 1A and 4). Expression of the fusion gene also made the cells sensitive to a PDGF receptor kinase inhibitor with regard to growth rate. These changes are all consistent with an autocrine PDGFBB stimulation. We demonstrated the expression of an in-frame COL1A1/PDGFB fusion transcript in B5/5 cells, identical to the fusion transcript produced by TNM2 tumor cells. Furthermore, this transcript is translated into the endoplasmic reticulum to form a COL1A1/PDGFB fusion peptide, with COL1A1 providing the signal peptide. This fusion peptide forms a disulfide-linked M-pocket homodimeric precursor protein that is further processed to Mf, M, 40,000 and M, 24,000 forms. The two latter forms appear identical to the M, 40,000 and M, 24,000 forms previously identified in studies on PDGFB-BB biosynthesis (28). The M, 40,000 form of PDGFB-BB is generated by cleavage between amino acid residues 81 and 82 of the PDGFB precursor and the M, 24,000 form represent a dimer of subunits that have undergone additional NH2- and COOH-terminal processing (28). We, thus, conclude that the COL1A1/PDGFB dimer is processed in a way that removes the entire COL1A1 portion of the fusion protein and that the final protein product(s) of the COL1A1/PDGFB fusion gene is a mature PDGFB-BB dimer.

We also demonstrated the activation of PDGF β-receptors in B5/5 cells but not in control cells (Fig. 1C), a finding that confirms that the COL1A1 fusion protein is processed to functional PDGFB-BB. Little or no PDGFB-BB is secreted from B5/5cells (Fig. 3), which is consistent with previous observations on PDGFB or v-sis-expressing cells (22, 28, 29). It is, thus, possible that, as in the case of v-sis-transformed cells, PDGF receptors are activated intracellularly and subsequently transported to the cell membrane, where they couple with the signal transduction machinery (30).

The tumors formed by B5/5 cells in nude mice were identical in growth, appearance, and collagen distribution as compared to tumors formed by PDGFB-expressing cells. This suggests that the COL1A1 part of the fusion protein does not contribute to the phenotype of the tumor. Most likely the COL1A1 part of the fusion gene thus serves to provide an active promoter and signal peptide for PDGFB. In addition, the translocation removes negative regulatory elements in the 5′.
end of the PDGFB gene and, thereby, potentiates protein production (31).

Experiments involving the PDGFB receptor tyrosine kinase inhibitor CGP57148B suggested that activation of PDGFB receptors was responsible for the transformation induced by COL1A1/PDGFB expression in NIH3T3 cells. This inhibitor reversed the transformed phenotype, giving B5/5 a morphology that was indistinguishable from that of the negative control CNEG-1 (Fig. 1). This inhibitor also reduced the growth of B5/5 derived tumors in nude mice (Fig. 4).

In conclusion, we show that the DFGSP/GCF-associated COL1A1/PDGFB fusion gene results in the production of a mature PDGFB-BB in a collagen-producing cell and thereby leads to autocrine growth stimulation. Expression of PDGFB β-receptors has been demonstrated in DFSP and GCF, both on tumor sections and on cultured tumor-derived cells (26, 32). A PDGFB autocrine loop, therefore, most likely occurs in DFSP/GCF tumor cells. On the basis of our findings, there seems to be a strong motivation to explore the possibility of targeting PDGFB receptors in pharmacological treatment of DFSP/GCF, for example, by investigating the effects of kinase inhibitors, like CGP57148B, on primary cultures of DFSP and GCF tumor cells.

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