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Biochemical and Biophysical Research Communications 308 (2003) 126–132
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A novel murine PDGF-D splicing variant results in significant differences in peptide expression and function☆

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Received 30 June 2003

Abstract

Platelet-derived growth factor (PDGF) is a potent mesenchymal cell mitogen and chemoattractant involved in the pathogenesis of fibroproliferative diseases. There are four known PDGF ligand isoforms designated A–D, two of which, C and D, were only recently discovered. We have identified a splicing variant in the PDGF-D isoform that occurs in mice, but not in humans. The presence of the splicing variant in murine PDGF-D appears to be due to an aberration in the splicing site at the junction of exons 5 and 6. The splicing variant results in a deletion predicted to have significant effects on peptide activity since it results in the deletion of bases within the cysteine knot domain that are important for peptide dimerization and receptor binding. It is important to appreciate differences between murine and human PDGF gene expression because PDGF is a key mitogen in the pathogenesis of fibrosis and mice are commonly employed as models for human disease.

Keywords: Mitogen; Fibrosis; Mouse; Exon

The platelet-derived growth factor (PDGF) family of peptides are potent mitogens and chemoattractants for mesenchymal cells, such as smooth muscle cells and fibroblasts [1]. Consequently, PDGF isoforms have been studied in the context of fibroproliferative diseases involving the vasculature, liver, kidney, dermis, and lung [2–6]. Both PDGF ligand and receptor expression are increased during fibrosis [6]. Moreover, various strategies aimed at blocking PDGF signal transduction limit the fibroproliferative response [7,8]. Thus, understanding the regulation of PDGF expression and activity is important to understanding fibrogenesis and wound healing.

There are now four known PDGF genes encoding polypeptides designated A–D and two cell membrane receptors designated alpha and beta. PDGF-A and -B chains were described over 30 years ago [1], whereas PDGF-C and -D remained undiscovered until 2000 [9,10]. PDGF-A and -B chains dimerize into homodimers, AA and BB, or an AB heterodimer. The ligand dimers bind to PDGF receptors, whereas monomers are inactive [11]. PDGF-C and -D chains also dimerize and bind to the PDGF alpha and beta receptors, but in contrast to PDGF-A and -B, require proteolytic cleavage for receptor binding [9,10]. PDGF-C and -D share substantial homology and so contain very similar protein domains. These regions include a signal sequence for secretion, a CUB domain that may serve to bind the polypeptide to connective tissue elements within the interstitium, an activation cleavage site, and a cysteine knot domain required for ligand dimerization and receptor binding [9,10].

PDGF-B and -C genes express a single mRNA, whereas a splicing variant has been described for PDGF-A chain involving exon 6 [12]. A small 18-base splicing variant has already been described for human PDGF-D [13]. In the manuscript we have identified a new splicing variant for PDGF-D that results in deletion

☆ *Abbreviations:* PDGF, platelet-derived growth factor; CUB, complement subcomponents Clr/Cls, Uegf, Bmp1; PCR, polymerase chain reaction; RIPA, radioimmunoprecipitation assay; DMEM, Dulbecco's modified Eagle's media; PBST, phosphate-buffered saline with Tween; SDS, sodium dodecyl sulfate; SSC, salt and sodium citrate; NIH, National Institutes of Health.

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doi:10.1016/S0006-291X(03)01346-9

of exon 6, which includes the cysteine knot domain that is crucial for dimerization and receptor binding.

Materials and methods

Antibodies. Antiserum against a synthetic peptide derived from mouse PDGF-D amino acid residues 202–216 (DPTLTADALDKTV AE) was generated using rabbits by Biosynthesis Inc. Antiserum was purified using an ImmunoPure Immobilized Protein A gel column (Pierce, USA). The murine monoclonal c-Myc (9E10) antibody was purchased from Santa Cruz (USA). Peroxidase-labeled goat anti-rabbit and goat anti-mouse antibodies were purchased from KPL (USA).

RT-PCR. Total tissue RNA was isolated from various mouse and human organs using the RNeasy Mini kit (Qiagen, Germany). Poly(A) RNA (0.6 μg) was reverse-transcribed at 55 °C using the ThermoScript RT-PCR system (Invitrogen, USA) in a 20 μl reaction mixture containing the oligo$(dT)_{20}$ primer, as described by the manufacturer. Two microliters of reaction was amplified by PCR in a mixture of 0.2 mM of each dNTP, 1× high fidelity PCR buffer, 2.0 mM $MgSO_4$, 0.2 μM primers and 1 U of Platinum Taq high-fidelity (Invitrogen, USA) in a final volume of 50 μl. Amplification was performed using the Eppendorf Mastercycler (Eppendorf Germany). The PCR products were separated on a 1.2% agarose gel and visualized by ethidium bromide staining.

The primers were designed to amplify the full-length mouse PDGF-D cDNA (upstream primer: 5′-ATG CAA CGG CTC GTT TTA GTC TCC ATT CTC C-3′, downstream primer: 5′-TTA TCG AGG TGG TCT TGA GCT GCA GAT ACA GTC-3′). The sequence of the downstream primer used for amplifying human PDGF-D was the same as that used for the mouse, because there is high homology for PDGF-D mRNA between human and mouse. The sequence of the upstream primer used on human cDNA was 5′-ATG CAC CGG CTC ATC TTT GTC TAC ACT CTA ATC-3′. The primers for mouse β-actin were purchased from Ambion.

RNase protection assay. The fragment of PDGF-D cDNA between the *Hae*II and *Eco*RI restriction sites was subcloned into the pBluescript KS(+) vector for use as a radiolabeled probe in the ribonuclease protection assay. The anti-sense RNA probe was labeled with [α-^{32}P]UTP (Perkin–Elmer, USA) using an in vitro transcription kit (Pharmingen, USA) and then was gel purified. The RNase protection assay was carried out with the RiboQuant Ribonuclease Protection Assay kit (Pharmingen) according to the instruction manual. Twenty micrograms of total RNA from each sample was used in this assay. Coincident assays for cyclophilin mRNA were performed to ensure the integrity and amount of each RNA sample (Ambion).

Dot blot. Genomic DNA from murine and human lung tissue was isolated using standard procedures [14]. Murine and human DNA were serially diluted and dotted onto nitrocellulose membranes (Schleicher & Schuell, USA). The membranes were baked at 80 °C for 30 min and hybridized with mouse and human ^{32}P-labeled (Perkin–Elmer, USA) full-length PDGF-D cDNA probes using the Prime-a-Gene labeling system (Promega, USA). The membranes were washed twice at 65 °C for 30 min with 2× SSC, 0.2% SDS, and twice with 0.2× SSC, 0.2% SDS. Finally, the blots were exposed to film and the signals were quantified using a phosphoimager (Fuji, Japan).

Expression of PDGF-D isoforms in NIH3T3 cells. The full-length and short murine PDGF-D cDNAs were amplified by PCR. The sequence of the upstream primer was 5′-GCG GGA TCC GCC ACC ATG CAA CGG CTC GTT TTA GTC TCC ATT CTC C-3′ and that of the downstream primer was 5′-ACG CGT CGA CTT CTC GAG GTG GTC TTG AGC TGC AGA TAC AGT C-3′. The PCR products were subcloned into the pCMV-Tag expression vector with a c-myc tag (Strategene, USA) for transfection experiments.

NIH 3T3 fibroblasts were plated at a density of 2.2×10^5 cells/well in 6-well plates and cultured in DMEM with 10% FBS for 24 h before transfection. Full-length and short PDGF-D expression plasmids were transfected into NIH 3T3 cells using Fugene 6 (Roche, USA). Cells were washed and cultured for another 48 h in serum-free media 24 h after transfection. Then 1 ml of the media was collected and concentrated to a volume of 30 μl using 10 kDa Microcon filters (Millipore, USA).

Western blot. Tissue from murine heart, kidney, and lung was homogenized in RIPA buffer (1× PBS, 1% NP-40, 0.5% deoxycholate, and 1% SDS) with a protease inhibitor cocktail (Sigma, USA). Eighty micrograms of protein from tissue or 12.5 μl of concentrated media were subjected to SDS–PAGE and transferred to PVDF membrane (Amersham, USA). The blot was blocked by 5% non-fat milk in PBST (0.05% Tween 20 in 1× PBS) for 1 h at room temperature, followed by incubation with primary antibody (1:200) for another hour at room temperature. After incubation, the blot was washed with PBST for 30 min and incubated with secondary antibody (1:10,000) for 1 h at room temperature. The blot was then washed with PBST for 1 h, developed using the ECL kit (Amersham), and exposed to X-ray film. For peptide blocking experiments, 5 μg/ml of the synthetic PDGF-D peptide, residues 202–216, was preincubated with the primary antibody.

Thymidine incorporation assay. Full-length, 18 bp-deleted and short PDGF-D cDNAs were individually subcloned into a pCMV-myc-tag expression vector. HeLa cells (4×10^5) (ATTC) were plated into 6-cm plates in DMEM with 10% FBS the day before transfection. Then the HeLa cells were transfected with the three PDGF-D plasmids in addition to a control plasmid lacking insert using Fugene 6 in DMEM with 2% FBS. Media collected 48 h after transfection was analyzed by Western analysis to confirm expression of the PDGF-D polypeptides and was assayed for fibroproliferative activity on MLF (mouse lung fibroblast) cells. An aliquot of the conditioned media was reserved for Western analysis of the HeLa conditioned media to confirm expression of the PDGF-D polypeptides. MLF cells (1×10^5) were seeded into 24-well plates in DMEM with 10% FBS overnight and then were rendered quiescent for 24 h in DMEM with 0.5% FBS. The cells were treated with 250 μl/well of transfected HeLa cell conditioned media for 36 h. Five μCi/ml of [^{3}H]thymidine (Amersham) was added to the cells 6 h after treatment. Finally, cells were washed and harvested with lysis buffer (0.2 N NaOH, 0.1% SDS) and radiolabeled thymidine incorporation was measured using a scintillation counter.

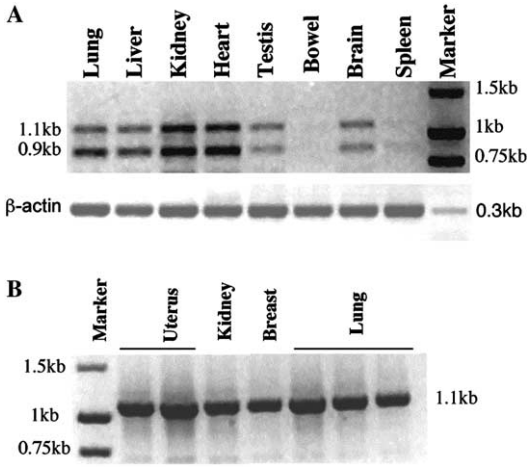

Fig. 1. Amplification of murine (A) and human (B) PDGF-D cDNA using total RNA from different organs. RT-PCR of murine RNA yielded a product of the predicted length and a shorter unexpected band, whereas human RNA yielded only the expected full-length product. β-Actin was used as an internal control. Among the organs we analyzed, the highest expression of both transcripts was found in heart and kidney. There was less expression in lung, liver, brain, and testis. No expression of either transcript was found in bowel.

For PDGF-D Western analysis, 1 ml of the same transfected HeLa cell media collected for the thymdine incorporation assay was incubated with 100 μl of heparin beads at 4 °C overnight. The beads were washed three times with 1× PBS and then boiled in 20 μl of 2× Laemmli buffer. The supernatants were loaded on 8–16% Tris–glycine mini-gel (Invitrogen) for Western blot using an anti-c-myc antibody as described above under the Western blot heading.

Statistical methods. All data are presented as means ± SEM. Unpaired two-tail *t* tests were performed using InStat 1.14 software. The results were considered to be statistically significant at $p < 0.05$.

Results

Identification of a murine PDGF-D splicing variant

Amplification of murine PDGF-D using RT-PCR resulted in the expected 1.1 kb product, plus an unexpected 0.9 kb product (Fig. 1A). In contrast, only the long PDGF-D PCR product was identified in human

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   1 atgcaacggc tcgttttagt ctccattctc ctgtgcgcga actttagctg ctatccggac
     M  Q  R  L   V  L  V   S  I  L   L  C  A  N   F  S  C   Y  P  D
 
  61 acttttgcga ctccgcagag agcatccatc aaagctttgc gcaatgccaa cctcaggaga
     T  F  A  T   P  Q  R   A  S  I  K  A  L  R   N  A  N   L  R  R
 
 121 gatgagagca atcacctcac agacttgtac cagagagagg agaacattca ggtgacaagc
     D  E  S  N   H  L  T   D  L  Y   Q  R  E  E   N  I  Q   V  T  S
 
 181 aatggccatg tgcagagtcc tcgcttcccg aacagctacc caaggaacct gcttctgaca
     N  G  H  V   Q  S  P   R  F  P   N  S  Y  P   R  N  L   L  L  T
 
 241 tggtggctcc gttcccagga gaaaacacgg atacaactgt cctttgacca tcaattcgga
     W  W  L  R   S  Q  E   K  T  R   I  Q  L  S   F  D  H   Q  F  G
 
 301 ctagaggaag cagaaaatga catttgtagg tatgactttg tggaagttga agaagtctca
     L  E  E  A   E  N  D   I  C  R   Y  D  F  V   E  V  E   E  V  S
 
 361 gagagcagca ctgttgtcag aggaagatgg tgtggccaca aggagatccc tccaaggata
     E  S  S  T   V  V  R   G  R  W   C  G  H  K   E  I  P   P  R  I
 
 421 acgtcaagaa caaaccagat taaaatcaca tttaagtctg atgactactt tgtggcaaaa
     T  S  R  T   N  Q  I   K  I  T   F  K  S  D   D  Y  F   V  A  K
 
 481 cctggattca agatttatta ttcatttgtg gaagatttcc aaccggaagc agcctcagag
     P  G  F  K   I  Y  Y   S  F  V   E  D  F  Q   P  E  A   A  S  E
 
 541 accaactggg aatcagtcac aagctctttc tctgggtgt cctatcactc tccatcaata
     T  N  W  E   S  V  T   S  S  F   S  G  V  S   Y  H  S   P  S  I
 
 601 acggacccca ctctcactgc tgatgccctg gacaaaactg tcgcagaatt cgataccgtg
     T  D  P  T   L  T  A   D  A  L   D  K  T  V   A  E  F   D  T  V
 
 661 gaagatctac ttaagcactt caatccagtg tcttggcaag atgatctgga gaatttgtat
     E  D  L  L   K  H  F   N  P  V   S  W  Q  D   D  L  E   N  L  Y
 
 721 ctggacaccc ctcattatag aggcaggtca taccatgatc ggaagtccaa aggtggacctg
     L  D  T  P   H  Y  R   G  R  S   Y  H  D  R   K  S  K   V  D  L
                                                              G
 781 gacaggctca atgatgatgt caagcgttac agttgcactc ccaggaatca ctctgtgaac
     D  R  L  N   D  D  V   K  R  Y   S  C  T  P   R  N  H   S  V  N
 841 ctcagggagg agctgaagct gaccaatgca gtcttcttcc cacgatgcct cctcgtgcag
     L  R  E  E   L  K  L   T  N  A   V  F  F  P   R  C  L   L  V  Q
 901 cgctgtggtg gcaactgtgg ttgcggaact gtcaactgga agtcctgcac atgcagctca
     R  C  G  G   N  C  G   C  G  T   V  N  W  K   S  C  T   C  S  S
 
 961 gggaagacag tgaagaagta tcatgaggta ttgaagtttg agcctggaca tttcaagaga
     G  K  T  V   K  K  Y   H  E  V   L  K  F  E   P  G  H   F  K  R
                                 I   E  V
1021 aggggcaaag ctaagaatat ggctcttgtt gatatccagc tggatcatca tgagcgatgt
     R  G  K  A   K  N  M   A  L  V   D  I  Q  L   D  H  H   E  R  C
1081 gactgtatct gcagctcaag accacctcga taa
     D  C  I  C   S  S  R   P  P  R
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Fig. 2. Nucleotide and deduced amino acid sequences of murine PDGF-D. RT-PCR products of murine PDGF-D cDNA were identified and sequenced. Nucleotide sequences are shown in lower case letters. Deduced amino acid sequences are shown in upper case letters below the nucleotide sequences. The exon borders are shown in italics and are underlined. Deleted sequences are shown in bold. There is a 215-bp-deletion corresponding to exon 6, which causes a frame shift and an early stop codon within exon 7 (underlined). There is also an 18-bp-deletion that does not result in a frame shift within a portion of the peptide that is cleaved away from the active ligand.

tissues (Fig. 1B). The genetic background of the mice used for Fig. 1A was C57BL/6, however, both the long and short PCR products were also identified in lung mRNA isolated from BALB/c mice, demonstrating that the presence of the PDGF-D short band in mice is not strain-specific (not shown). Among the different murine organs we detected high expression of both PDGF-D transcripts in heart and kidney and medium expression in lung, liver, brain, and testis. There was very weak expression of both PDGF-D transcripts in spleen and neither transcript was expressed in the intestines (Fig. 1A). The ratio of the long and short transcripts was similar in the various organs tested.

Sequences of PDGF-D splicing variants

The long and short PCR products were cloned into expression vectors using primers containing restriction sites and the DNA sequence of the PDGF-D fragments was determined. The results of the sequencing are shown in Figs. 2 and 3. The larger PCR product was a combination of two sequences differing by an 18-base deletion in the CUB domain. The 18-base deletion does not alter the reading frame and lies in a portion of the peptide that is cleaved away upon PDGF activation. The short PCR product was identical to the long product with the exception that there is deletion of all 215 bases corresponding to exon 6 of PDGF-D. The short PDGF-D product was also comprised of two sequences differing by an 18-base deletion in the CUB domain.

Confirmation of the short PDGF-D transcript

A ribonuclease protection assay probe was designed to differentiate between the long and short PDGF-D mRNA moieties. Fig. 4 depicts the results of an RPA for PDGF-D on RNA isolated from murine heart, liver, kidney and lung. The 254 base upper band corresponds to the expected product from the long PDGF-D mRNA, and the 127 base band corresponds to the expected product from the short PDGF-D mRNA. The ratio of long to short PDGF-D mRNA expression was similar in all four tissues that were analyzed and taking the relative sizes of the products into account was found to be 1:1.8. The results from this assay confirmed the presence of the short PDGF-D message.

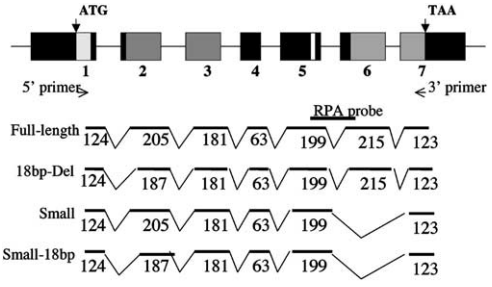

Fig. 3. Schematic diagram of the PDGF-D gene and alternative mRNA transcripts. The region of the signal peptide is stippled, the CUB domain is shown in dark gray, the proteolytic cleavage site is shown in white, and the cysteine knot growth factor receptor-binding domain is shown in light gray. The models of the four transcripts are shown below the gene structure. All of exon 6 is deleted in the small transcript. The probe for the RNase protection assay, shown in Fig. 4, is from nucleotide 646 to 900 and spans portions of exons 5 and 6.

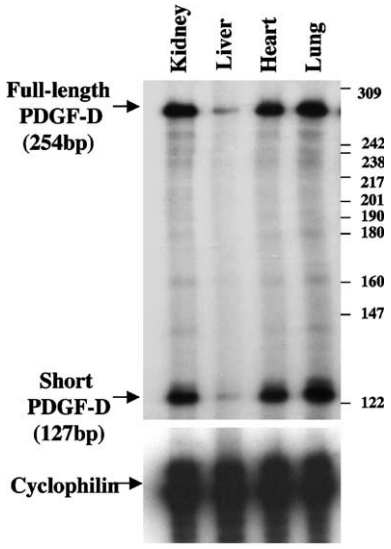

Fig. 4. Confirmation of the small transcript of murine PDGF-D mRNA by RPA. Total RNA from different mouse organs was hybridized with a ^{32}P-labeled RPA probe spaning portions of exons 5 and 6, and then digested with RNase. The final products were separated on a 5% denaturing polyacrylamide gel and exposed to film. Two products of the sizes predicted by sequencing the murine PDGF-D PCR products were identified, confirming the existence of the short PDGF-D mRNA transcript. The ratio of the long transcript vs. short using a phosphoimager for quantification is about 1:1.8 in all four different organs.

A dot blot comparison was made between human and murine DNA to determine whether mice have only one copy of the PDGF-D gene, as is reported for humans [15]. The probes employed for the results depicted in Fig. 5 were of similar specific activity. The blot signal

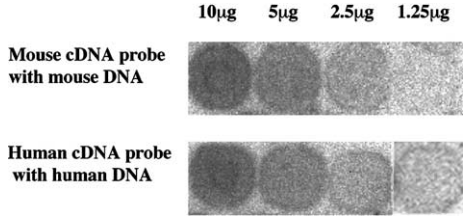

Fig. 5. Comparison of gene copy number for murine and human PDGF-D genes by dot blot. Murine and human genomic DNA were serially diluted and dotted onto two separate nitrocellulose membranes. The blots were hybridized to murine or human ^{32}P-labeled cDNA probes of similar specific activity. The result was analyzed using a phosphoimager and indicates that, as per the human PDGF-D gene, there is only one copy of the murine PDGF-D gene.

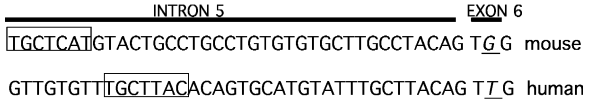

Fig. 6. A comparison of the sequences for murine and human PDGF-D at the border of intron 5 and exon 6. The lariat acceptor consensus sequence is labeled in the box. The acceptor sequences in exon 6 differ between mouse and human (underlined).

intensity for mouse DNA was equal to that of human DNA, indicating that mice also have only one copy of the PDGF-D gene. Mouse and human data bases were searched to identify the sequence of the intron just proximal to the 5′ end of exon 6 and are shown in Fig. 6. The results indicate the mice have a poor sequence for splicing [16] that could account for the presence of the short PDGF-D mRNA transcript observed in mice, but not in humans.

Expression of the short PDGF-D peptide

We could not employ anti-PDGF-D antibodies used by other investigators for our experiments because their antibodies bind to regions that are deleted in the peptide derived from the short mRNA transcript. Hence, a rabbit anti-mouse PDGF-D polyclonal antibody was generated against PDGF-D amino acids 202–216, which are located at the 3′ end of exon 5. Full-length, 18-base deletion, and short PDGF-D transcripts were subcloned into vectors with a c-myc tag on the 3′ prime end. However, there is a premature stop codon in exon 7 in the short transcript that is caused by a frame shift that prevented the translation of the c-myc tag. The three PDGF-D constructs were transfected into NIH3T3 cells and the media was collected 48 h after transfection. Also 80 μg of total protein from mouse heart, lung, and kidney were subjected to Western analysis. The full-length and 18 bp deleted forms of PDGF-D were detected by the PDGF-D antibody, and bands of the same size were also bound by the c-myc antibody as expected (Fig. 7). The short form of PDGF-D was also bound by the PDGF-D antibody, as a polypeptide of the expected size was detected in cells transfected with the short PDGF-D construct. Specificity was also confirmed by the results of part B of this figure showing that the binding of the PDGF-D antibody to the 55 and 43 kDa bands is inhibited by preincubation of the antibody with the PDGF-D synthetic peptide used to raise the antibody. The long, but not the short PDGF-D peptide, was identified in murine heart, and neither the long or short PDGF-D peptides were detected in kidney or lung tissues (not shown). Thus, murine heart, kidney, and lung do not appear to express the short PDGF-D peptide in quantities sufficient to be detected by Western analysis.

Mitogenic activity of the full length, 18-base deletion and short PDGF-D constructs

To examine the functional consequences of the lack of exon 6, PDGF-D splice variants were transfected into HeLa cells, and conditioned media were assayed for mitogenic activity on mouse lung fibroblast. The full length and 18-base deletion PDGF-D constructs were potent inducers of fibroblast proliferation, inducing

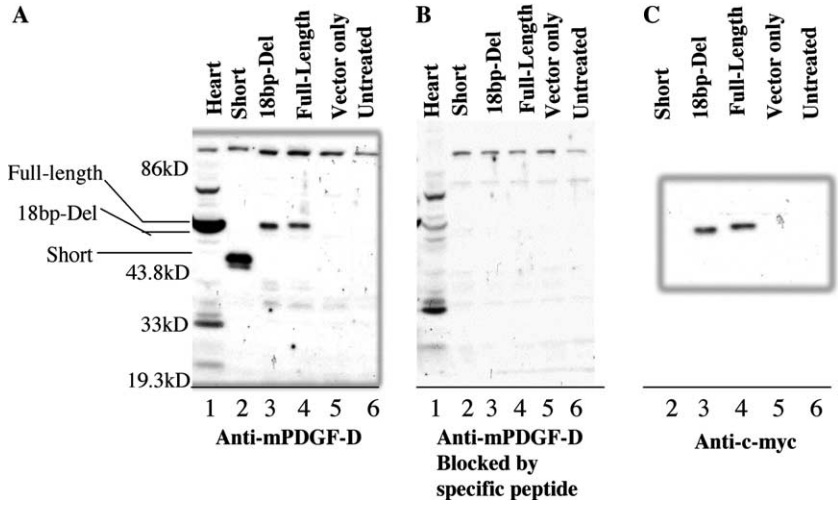

Fig. 7. Expression of PDGF-D polypeptides. A rabbit anti-mouse PDGF-D polyclonal antibody was generated against PDGF-D amino acids 202–216. PDGF-D transcripts were subcloned into vectors with a c-myc tag on the 3′ prime end. However, there is a stop codon in exon 7 in the short transcript that prevented the translation of the c-myc tag. Lanes 2–6 show immunoblots of concentrated media from NIH 3T3 cells transfected with the indicated expression construct. Lane 1 is 80 μg of total protein from murine heart. (A) Western blot using the anti-mouse PDGF-D antibody. (B) Western blot using anti-mouse PDGF-D antibody preincubated with 5 μg/ml of the specific peptide used to generate the antibody. (C) Western blot of media from transfected NIH 3T3 cells using an anti-c-myc antibody, shown as a control for the new anti-PDGF-D antibody. The long, but not the short PDGF-D peptide, was identified in murine heart.

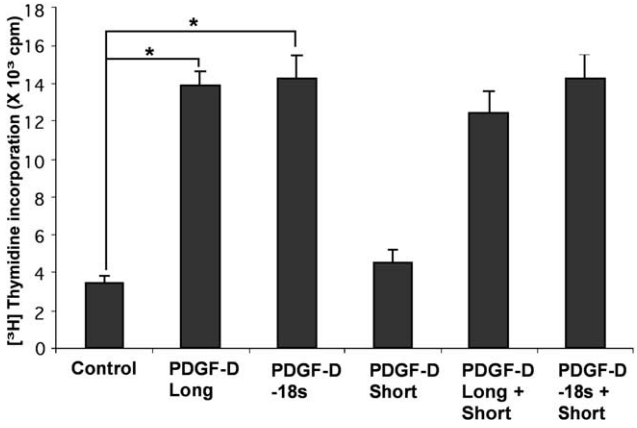

Fig. 8. Mitogenic activity of PDGF-D splicing variants. HeLa cells were transfected with control plasmid and plasmids encoding PDGF-D splicing variants, and conditioned media were assayed for mitogenic activity by [^{3}H]thymidine incoporation in mouse lung fibroblasts. The results are represented as means ± SEM ($n = 3$). *, $p < 0.01$ vs. control plasmid.

significant and similar increases in thymidine incorporation over the control vector (control: 3446 ± 200; PDGF-D Long: 13,900 ± 469; PDGF-D -18s: 14,300 ± 704; $p < 0.01$) (Fig. 8). In contrast, the short PDGF-D construct demonstrated no difference in mitogenic activity in comparison with the control plasmid (PDGF-D Short: 4492 ± 397). Cotransfection of the short PDGF-D plasmid with the active PDGF-D constructs did not diminish the potency of the full length or 18-base deletion PDGF-D plasmids. A Western blot from the transfected HeLa cell conditioned media demonstrated similar band intensity for all three polypeptide species expressed from the PDGF-D plasmids (not shown), demonstrating that the results shown in Fig. 8 are not due to differences in PDGF-D polypeptide expression.

Discussion

We have found that murine PDGF-D has two splicing variants. One variant is an 18 base deletion in the CUB domain, similar to that described in humans [13]. In addition, murine, but not human, PDGF-D has a more significant splicing variant involving deletion of exon 6, which causes a frame shift resulting in an early termination codon in exon 7. The biological significance or consequence of the 18 base deletion remains unknown except for the fact that we and other investigators have determined that PDGF-D is biologically active with or without the 18 base deletion (Fig. 8) [13]. The splicing variant that we have identified results in a deletion that we would predict to have significant effects on peptide activity since it results in deletion of regions within the cysteine knot domain that are important for peptide dimerization and receptor binding [9,10].

The presence of the short mRNA moiety lacking exon 6 was confirmed using a ribonuclease protection assay (Fig. 4). We were unable to detect the short PDGF-D band using PCR in human tissues. Our data also indicate that, similar to humans [15], mice have only one copy of the PDGF-D gene (Fig. 5). To determine the cause for the short splicing variant, sequences of the murine and human introns at the 5′ end of exon 6 were examined (Fig. 6). The sequence for the human splicing site is more compatible, compared with that of the mouse, with published optimal splicing sequences [16]. Poor use of the suboptimal exon 6 splice site in the mouse likely accounts for the presence of the short mRNA construct in mice, but not in humans.

An anti-PDGF-D antibody and a plasmid with the short PDGF construct were produced to determine whether the short PDGF-D mRNA is translated into a polypeptide. We do not know the reason why we are able to detect the short peptide in a transfected murine cell line, but not in any of the three murine tissues we tested. We have considered instability of the short polypeptide as an explanation. The short band may be more strongly expressed in the NIH3T3 cells due to higher expression with transfection or greater stability due to differences in the 3′ untranslated region in native short PDGF-D in comparison to the plasmid construct.

Splicing variants in mice that are not observed in humans have been described for other key molecules such as p53 [17]. It is imperative to be *cognizant* of these splicing variants when using animal models to understand human diseases. Consequently, it is important to understand the significance of splicing variants identified in potent mesenchymal cell mitogens, such as the PDGF isoforms. Sequencing of the genome has revealed much less complexity than expected. Alternative splicing provides an essential mechanism to generate the diversity of gene expression required from a limited number of genes. One model relevant to the findings here is the alternative splicing of the VEGF mRNA that generates a protein that appears to negatively regulate VEGF function [18].

Splicing variants have been described for PDGF-A, but unlike our observations with PDGF-D, the PDGF-A splicing variant is observed in humans as well as mice [12,19]. We undertook the analysis of the PDGF-D splicing variant because PDGF-A-chain splicing affects its localization and biological activity [20]. Both long and short PDGF-A peptides are biologically active; however, there are some significant differences regarding conditions of expression and modulation of biological activity that are attributed to the presence of the deletion. The short form of PDGF-A is expressed in quiescent and active cells, but the long form of PDGF-A is predominantly secreted by activated cells [21]. The short PDGF-A isoform is excreted into the media, whereas the long PDGF-A isoform contains a highly basic

carboxy terminal extension that binds the peptide to heparin-like matrix constituents near the cell surface [22]. Binding of the long PDGF-A isoform to the matrix could result in a prolonged stimulation of adjacent cells [23]. In contrast, we were unable to identify the short PDGF-D polypeptide in murine tissues and our data indicates (Fig. 8) that the short PDGF-D construct is not a fibroblast mitogen and does not inhibit the mitogenic activity of the full-length or 18-base deletion PDGF-D polypeptides in vitro.

In summary, both PDGF-A and PDGF-D have splicing variants involving all of exon 6. However, exon 6 for PDGF-D includes the cysteine knot domain, and its deletion causes a frame shift leading to early termination in exon 7. In contrast, the deletion of exon 6 in PDGF-A does not result in a frame shift and does not involve critical regions of the cysteine knot that are crucial for ligand dimerization and receptor binding. The short form of murine PDGF-D does not appear in isolates from tissues expressing abundant amounts of PDGF-D. Thus, at this juncture the short PDGF-D splicing variant does not appear to be a mechanism for localizing or modulating PDGF-D expression, as occurs for PDGF-A. The presence of the short PDGF-D isoform appears to be due to a murine aberration in the splicing site in the intron between exons 5 and 6.

References

[1] E.W. Raines, Platelet-derived growth factor, in: Peptide Growth Factors and Their Receptors, in: M.B. Sporn, A.B. Roberts (Eds.), Handbook of Experimental Pharmacology, Springer, Berlin, 1990, pp. 419–472.
[2] C.H. Heldin, B. Westermark, Mechanism of action and in vivo role of platelet-derived growth factor, Physiol. Rev. 79 (1999) 1283–1316.
[3] S.L. Friedman, Closing in on the signals of hepatic fibrosis, Gastroenterology 112 (1997) 1406–1409.
[4] C. Betsholtz, E.W. Raines, Platelet-derived growth factor: a key regulator of connective tissue cells in embryogenesis and pathogenesis, Kidney Int. 51 (1997) 1361–1369.
[5] K. Takehara, Growth regulation of skin fibroblasts, J. Dermatol. Sci. 24 (Suppl. 1) (2000) S70–77.
[6] J.A. Lasky, A.R. Brody, Interstitial fibrosis and growth factors, Environ. Health Perspect. 108 (Suppl. 4) (2000) 751–762.
[7] A.B. Rice, C.R. Moomaw, D.L. Morgan, J.C. Bonner, Specific inhibitors of platelet-derived growth factor or epidermal growth factor receptor tyrosine kinase reduce pulmonary fibrosis in rats, Am. J. Pathol. 155 (1999) 213–221.
[8] E.A. Kallio, P.K. Koskinen, E. Aavik, E. Buchdunger, K.B. Lemstrom, Role of platelet-derived growth factor in obliterative bronchiolitis (chronic rejection) in the rat, Am. J. Respir. Crit. Care Med. 160 (1999) 1324–1332.
[9] X. Li, A. Ponten, K. Aase, L. Karlsson, A. Abramsson, M. Uutela, G. Backstrom, M. Hellstrom, H. Bostrom, H. Li, P. Soriano, C. Betsholtz, C.H. Heldin, K. Alitalo, A. Ostman, U. Eriksson, PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor, Nat. Cell Biol. 2 (2000) 302–309.
[10] W.J. LaRochelle, M. Jeffers, W.F. McDonald, R.A. Chillakuru, N.A. Giese, N.A. Lokker, C. Sullivan, F.L. Boldog, M. Yang, C. Vernet, C.E. Burgess, E. Fernandes, L.L. Deegler, B. Rittman, J. Shimkets, R.A. Shimkets, J.M. Rothberg, H.S. Lichenstein, PDGF-D, a new protease-activated growth factor, Nat. Cell Biol. 3 (2001) 517–521.
[11] C.H. Heldin, B. Westermark, Signal transduction by the receptors for platelet-derived growth factor, Science 96 (1990) 193–196.
[12] T. Collins, D.T. Bonthron, S.H. Orkin, Alternative RNA splicing affects function of encoded platelet-derived growth factor A chain, Nature 328 (1987) 621–624.
[13] T. Hamada, K. Ui-Tei, J. Imaki, F. Takahashi, H. Onodera, T. Mishima, Y. Miyata, The expression of SCDGF/PDGF-C/fallotein and SCDGF-B/PDGF-D in the rat central nervous system, Mech. Dev. 112 (2002) 161–164.
[14] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning, second ed., New York, 1989.
[15] M. Uutela, J. Lauren, E. Bergsten, X. Li, N. Horelli-Kuitunen, U. Eriksson, K. Alitalo, Chromosomal location, exon structure, and vascular expression patterns of the human PDGFC and PDGFD genes, Circulation 103 (2001) 2242–2247.
[16] S.M. Mount, Genomic sequence, splicing, and gene annotations, Am. J. Hum. Genet. 67 (2000) 788–792.
[17] M. Laverdiere, J. Beaudoin, A. Lavigueur, Species-specific regulation of alternative splicing in the C-terminal region of the p53 tumor suppressor gene, Nucleic Acids Res. 28 (2000) 1489–1497.
[18] D.O. Bates, T.G. Cui, J.M. Doughty, M. Winkler, M. Sugiono, J.D. Shields, D. Peat, D. Gillatt, S.J. Harper, VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma, Cancer Res. 62 (2002) 4123–4131.
[19] F. Rorsman, C. Betsholtz, Characterization of the mouse PDGF A-chain gene. Evolutionary conservation of gene structure, nucleotide sequence and alternative splicing, Growth Factors 6 (4) (1992) 303–313.
[20] F. Lustig, J. Hoebeke, G. Ostergren-Lunden, F. Velge-Roussel, G. Bondjers, U. Olsson, U. Ruetschi, G. Fager, Alternative splicing determines the binding of platelet-derived growth factor (PDGF-AA) to glycosaminoglycans, Biochemistry 35 (1996) 12077–12085.
[21] N. Fukuda, H. Kishioka, C. Satoh, T. Nakayama, Y. Watanabe, M. Soma, Y. Izumi, K. Kanmatsuse, Role of long-form PDGF A-chain in the growth of vascular smooth muscle cells from spontaneously hypertensive rats, Am. J. Hypertens. 10 (1997) 1117–1124.
[22] R.A. Pollock, W.D. Richardson, The alternative-splice isoforms of the PDGF A-chain differ in their ability to associate with the extracellular matrix and to bind heparin in vitro, Growth Factors 7 (1992) 267–277.
[23] A. Krettek, G. Fager, H. Lindmark, C. Simonson, F. Lustig, Effect of phenotype on the transcription of the genes for platelet-derived growth factor (PDGF) isoforms in human smooth muscle cells, monocyte-derived macrophages, and endothelial cells in vitro, Arterioscler. Thromb. Vasc. Biol. 17 (1997) 2897–2903.