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Inhibition of PDGF BB stimulated DNA synthesis in rat aortic vascular smooth muscle cells by the expression of a truncated PDGFβ receptor

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Rat aortic vascular smooth muscle cells (VSMCs) were transfected with a vector encoding a truncated human PDGFβ receptor or the full-length human PDGFβ receptor. Cells stably expressing the truncated human PDGFβ receptor or the full-length human PDGFβ receptor were selected and the effect of PDGF BB on DNA synthesis in these cells was studied. Cells expressing the full-length PDGFβ receptor entered DNA synthesis normally, however, cells expressing the truncated PDGFβ receptor failed to enter DNA synthesis in response to PDGF BB. These data show that the mitogenic response of rat aortic vascular smooth muscle cells to PDGF BB can be inhibited by the expression of a truncated PDGFβ receptor.

Platelet-derived growth factor; Truncated receptor; Vascular smooth muscle cell

1. INTRODUCTION

The role of platelet-derived growth factor (PDGF) in the migration and proliferation of vascular smooth muscle cells (VSMCs) following endothelial injury remains uncertain [1,2]. To establish any role in vivo for PDGF it is necessary either to neutralise the endogenous PDGF or to block the receptor through which it signals. PDGF initiates mitogenesis through its interaction with the extracellular domain of the PDGF receptor [3,4]. This interaction has been shown to result in the dimerisation of PDGF receptor monomers with a subsequent increase in tyrosine kinase activity [5–7]. The monomers of the complex cross-phosphorylate each other on tyrosine residues [8] and this leads to the binding to the PDGF receptor dimer and tyrosine phosphorylation of other substrates (e.g. GTPase activating protein, phosphatidylinositol-3-kinase and phospholipase Cγ [7,9–13]).

Ueno et al. [14] demonstrated that a truncated PDGFβ receptor lacking most of the cytoplasmic domain would form a non-functional dimer with the wild type PDGFβ receptor upon ligand binding. Expression of a truncated PDGFβ receptor in VSMCs may therefore provide a mechanism by which the PDGF signalling pathway can be inhibited sufficiently to prevent DNA synthesis in response to PDGF BB.

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Abbreviations: PDGF, platelet-derived growth factor; VSMC, vascular smooth muscle cell; DMEM, Dulbecco's modification of Eagle's medium; FCS, foetal calf serum; BSA, bovine serum albumin; BrdU, bromodeoxyuridine.

The aim of this study was to determine whether ectopic expression of a truncated PDGF β receptor in rat aortic VSMCs could inhibit PDGF BB-mediated cellular events in vitro. A gene encoding the human PDGFβ receptor truncated in the tyrosine kinase 2 domain was generated and expressed in cultured rat aortic VSMCs and the effect of expression of this gene on DNA synthesis was studied.

2. MATERIALS AND METHODS

2.1. *Generation and sequencing of truncated PDGF receptor vector*

A vector for expression of a truncated PDGFβ receptor in VSMCs was constructed from the vector PDGFRβSV7D [15]. This plasmid was digested with *Bam*HI and a 5.3 kb fragment containing the N-terminal portion of the receptor cDNA was isolated from low melting point agarose. The DNA fragment was then recircularised and, following transformation, an appropriate clone was selected by plasmid mapping. The sequence was confirmed by cloning an *Eco*RI–*Hpa*I fragment into bluescript KS+ and sequencing the new join between the PDGF receptor cDNA and the SV40 termination sequence using double stranded dideoxy sequencing. A diagramatic representation of the proteins coded for by the full-length and truncated receptor vectors is given in Fig. 1. All procedures were carried as described in Sambrook et al. [16]. All the enzymes used in cloning were obtained from LKB-Pharmacia.

2.2. *Transfection and selection of cells*

Subcultured VSMCs from adult rat aortae were derived as described [17] grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with penicillin and streptomycin + 10% foetal calf serum (FCS) and passaged 1:2 every 4 days. For transfection, cells between the 8th and 10th passage were grown to approximately 80% confluence then harvested by trypsinisation and the trypsin quenched by resuspending the cells in DMEM + 10% FCS. The cells were spun down and resuspended in electroporation buffer (NaCl, 137 mM; KCl, 5 mM; Na_2HPO_4, 0.7 mM; HEPES, 25 mM (pH 7.05); dextrose 6 mM; and salmon sperm DNA, 300 $\mu g \cdot ml^{-1}$) at 3×10^6 cells $\cdot ml^{-1}$. The suspension (0.8 ml) was put into electroporation cuvettes (Bio-Rad)

with 40 μg of PDGFβ receptor plasmid DNA and 5 μg of SV2neo DNA and electroporated at 280 V and 960 μF. The cells were allowed to recover for 10 min before being added to DMEM + 10% FCS (9.2 ml) and plated into 75 cm^2 flasks. Approximately 12% of the cells survive this electroporation procedure. Cells were grown in 400 $\mu g \cdot ml^{-1}$ geneticin for two weeks before the geneticin concentration was reduced to 200 $\mu g \cdot ml^{-1}$. Cells were subcultured every week for the first 3 weeks and then every 4 days.

2.3. *Immunocytochemistry and Northern analysis*

Cells were plated into 96-well plates for immunocytochemistry or onto 13 mm glass coverslips for immunofluorescence and grown for 2 days. After a 6 h serum deprivation the cells were washed 3 times with phosphate-buffered saline (PBS: NaCl, 137 mM; Na_2HPO_4, 8.1 mM; KH_2PO_4, 1.5 mM; KCl, 2.7 mM pH 7.4) before being fixed with 4% paraformaldehyde in PBS for 10 min. After fixing the cells were washed 3 times for 3 min with PBS before incubation with anti human PDGF β receptor antibody (PR7212) [18] at 40 $\mu g \cdot ml^{-1}$ in PBS + 3% bovine serum albumin (BSA, Sigma) overnight. After washing with PBS (3 times for 3 min), cells for peroxidase staining were incubated with peroxidase conjugated antimouse IgG antibody raised in goat (Sigma), diluted 1:200 in PBS + 3% BSA, for 2 h prior to washing and staining with diaminobenzidine. Cells for immunofluorescence were incubated with biotin conjugated antimouse IgG raised in goat (Sigma) diluted 1:500 for 2 h. After washing the cells (3 times for 3 min with PBS) fluoroscein isothiocyanate conjugated to streptavidin was added in PBS + 3% BSA for 1 h. The cells were then washed in PBS and photographed using a Nikon epifluorescence microscope linked to a Joyce Loebel image analysis system.

Cytoplasmic RNA extraction and Northern analysis was carried out as described in Kemp et al. [19] using the cDNA of the extracellular domain of the human PDGF receptor as a probe.

2.4. *DNA synthesis*

DNA synthesis was assayed by bromodeoxyuridine (BrdU) incorporation using Amersham Kit RPN 20. Cells were subcultured 1:4 into 96-well plates and grown for 2 days prior to being washed 3 times in PBS and put into serum free DMEM for 48 h. Cells were stimulated with recombinant PDGF BB (recombinant homodimer, Penninsular) at 0, 10, 100 and 200 $ng \cdot ml^{-1}$ or 10% FCS. Bromodeoxyuridine was added 18 h after stimulation and the cells were fixed and stained 6 h later. This period was chosen because it coincided with peak DNA synthesis in these cells in response to PDGF BB. The assay was carried out as described in the manufacturers instructions.

3. RESULTS AND DISCUSSION

3.1. *Expression of the full-length and truncated human PDGF receptors*

Expression of the full-length and truncated human PDGFβ receptors in rat aortic VSMCs after 3 weeks of selection in geneticin, was demonstrated by Northern analysis and immunofluorescence. Northern analysis of RNA from cells transfected with the truncated PDGFβ receptor gene showed two RNA species hybridising to a cDNA encoding the extracellular domain of the human PDGFβ receptor, one of approximately 5 kb,

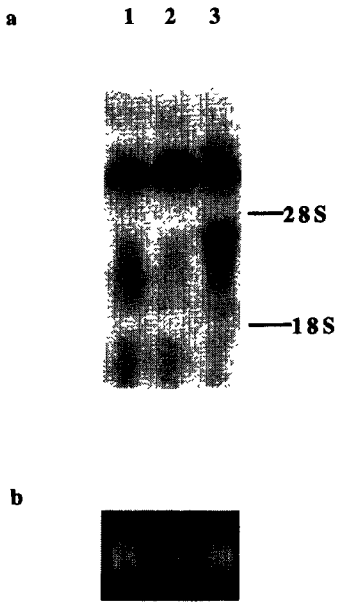


Fig. 2. (a) Northern analysis of cytoplasmic RNA from transfected VSMCs. RNA was prepared as described in section 2 and probed using the a cDNA encoding the extracellular domain of the human PDGF receptor. Lane 1, VSMCs transfected with SV2neo alone show one band of approximately 5 kb due to expression of the rat receptor gene; lane 2, VSMCs transfected with the full-length PDGFβ receptor gene and SV2neo show one band of approximately 5 kb due to expression of both the full-length human and rat receptor genes; lane 3, VSMCs transfected with the truncated PDGFβ receptor gene and SV2neo show two bands one of approximately 5 kb due to expression of the full-length rat receptor gene and one of approximately 3 kb due to expression of the truncated human receptor gene. The positions of the 18S and 28S rRNAs are marked. (b) Ethidium bromide staining of the 18S rRNA in the gel prior to transfer.

similar to that found in RNA from transfected rat aortic VSMCs transfected with the SV2neo vector alone, and one of approximately 3 kb corresponding to the expected size of the truncated human PDGFβ receptor mRNA (Fig. 2). Analysis of RNA from cells transfected with the full-length human receptor showed one broad band at approximately 5 kb due to both human full-length and rat wild type PDGFβ receptor gene expression. Expression of the endogenous rat receptor was not affected by transfection with the truncated PDGFβ receptor vector and SV2neo compared with transfection with SV2neo alone (Fig. 2). In cells transfected with the full-length receptor vector and SV2neo the amount of PDGFβ receptor mRNA was increased, relative to total RNA levels, presumably due to expression both the human and rat receptor genes (Fig. 2).

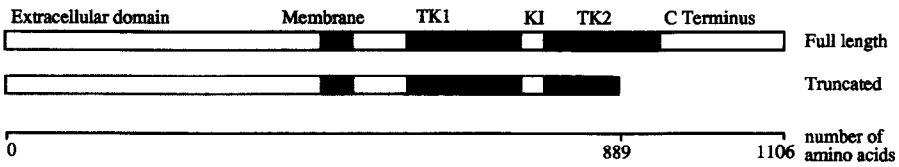
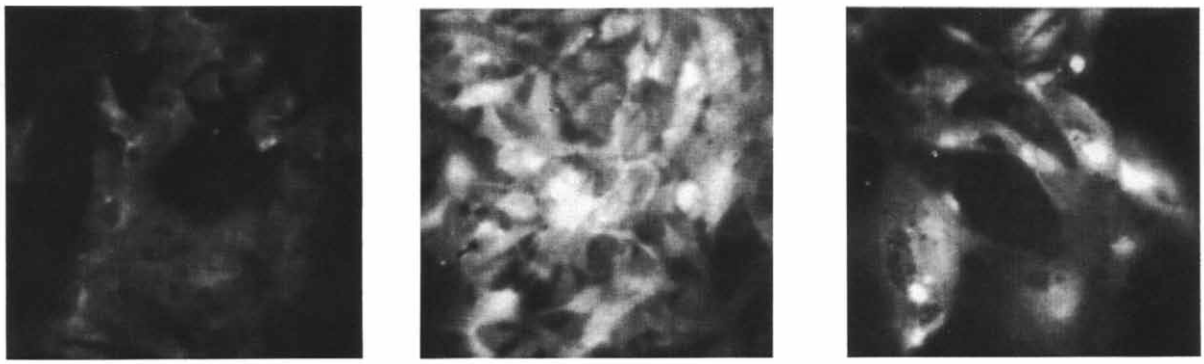


Fig. 1. Size and domain composition of the receptor constructs. TK1, tyrosine kinase 1 domain; TK2, tyrosine kinase 2 domain; KI, kinase insert sequence.



a b c

Fig. 3. Immunofluorescence of rat aortic VSMCs expressing the human PDGFβ receptor. (a) VSMCs transfected with SV2neo alone. (b) VSMCs transfected with the truncated PDGFβ receptor gene and SV2neo. (c) VSMCs transfected with the full-length PDGFβ receptor gene and SV2neo.

The production of the human PDGFβ receptor protein was examined by immunocytochemical staining of cell surface proteins using an anti-human PDGFβ receptor antibody (PR7212, [18]) which recognises the human PDGFβ receptor with much higher affinity than the rat PDGFβ receptor. Cells expressing either the full-length human PDGFβ receptor or the truncated PDGFβ receptor showed increased binding of the antibody PR7212 compared with wild-type rat cells (Fig. 3). These studies indicated that both the full-length and truncated human PDGFβ receptor mRNAs were translated. Furthermore, since the cells were not permeabilised by the fixation procedure only transfected receptor proteins localised on the cell surface were detected. Thus the data indicate that the rat cells correctly processed and targetted the human receptor protein to the cell surface (Fig. 3).

3.2. *Effects of the truncated and full-length human receptors on DNA synthesis in VSMCs*

The DNA synthesis response of subcultured rat aortic VSMCs to stimulation with PDGF BB stimulation was determined by BrdU incorporation into DNA (Fig. 4). The number of nuclei synthesizing DNA during the BrdU pulse increased in a dose dependent manner in response to PDGF BB reaching half-maximal stimulation at approximately 7 ng · ml^{-1} and maximal stimulation between 10 and 50 ng · ml^{-1} (Fig. 4). This is consistent with the data of Kawahara et al. [20] who found half maximal stimulation of DNA synthesis by 4 ng · ml^{-1} PDGF BB and maximal stimulation by 25 ng · ml^{-1} in VSMCs. Transfection of the cells with the SV2neo vector alone and selection of the cells in geneticin showed a similar response to PDGF BB to the untransfected VSMCs but had a slightly increased background level of DNA synthesis (17% in the absence of added mitogen increasing to 36% in the presence of 10 ng · ml^{-1} PDGF BB).

VSMCs expressing the truncated PDGFβ receptor were stimulated with PDGF BB or 10% FCS. Fig. 5 shows that in the absence of added mitogen approximately 13 ± 1.2% of the VSMCs expressing the truncated PDGFβ receptor were in DNA synthesis during the BrdU pulse. This proportion was not increased by the addition of up to 200 ng · ml^{-1} PDGF BB. By contrast, in the presence of 10% FCS 60 ± 3% of the cells were in DNA synthesis during the same period. These observations indicate that the cells were unable to respond to PDGF BB although they were able to synthesize DNA in response to serum. The data therefore suggest that sufficient truncated receptor was produced by the cells to prevent the normal signal transduction response to PDGF BB. Since there was no increase in the percentage of cells in DNA synthesis at concentrations of PDGF BB up to 200 ng · ml^{-1} the major mechanism of inhibition of PDGF signalling was likely to

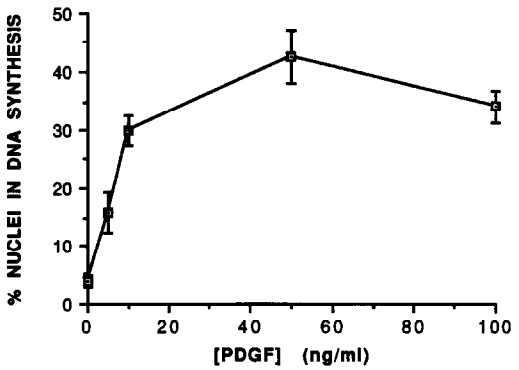


Fig. 4. DNA synthesis dose response curve for untransfected VSMCs to PDGF BB. Passaged VSMCs were subcultured 1:4 into 96 well plates and grown for 2 days. The cells were then washed with serum free DMEM and incubated for 48 h in the absence of serum. The cells were then restimulated with serum free DMEM + PDGF (0, 5, 10, 50, 100 ng · ml^{-1}). BrdU was added to the cells for 6 h between 18 and 24 h after restimulation. The cells were fixed and stained for BrdU incorporation at the end of the BrdU pulse. Data are presented as mean ± S.E.M. (n = 4 counts of BrDu stained nuclei).

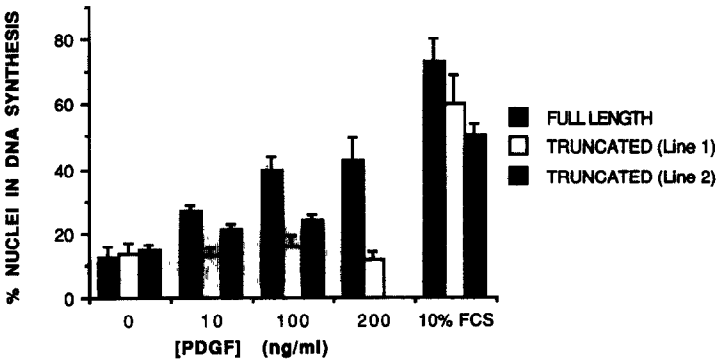


Fig. 5. DNA synthesis dose response for transfected VSMCs to PDGF BB. VSMCs stably expressing the full-length or truncated human PDGFβ receptor quiesced as described above were restimulated with PDGF BB (at 0, 10 100 and 200 ng · ml^{-1}) or 10%FCS and DNA synthesis was assayed by the incorporation of BrdU between 18 and 24 h as described in section 2. Data are presented as mean ± S.E.M. (n = 4 counts of BrdU stained nuclei). Data are typical of 3 separate experiments. Cells from truncated line 2 were only stimulated at 0, 10 and 100 ng · ml^{-1} PDGF BB.

have been heterodimer formation of the truncated receptor with the wild type receptor, causing a reduction in V_{max} by competition of the inactive receptor for the available PDGF BB and not by depletion of the PDGF BB from the medium which would have resulted in an increase in apparent K_m.

In the absence of added mitogen the proportion of cells expressing the full-length human PDGFβ receptor which were in DNA synthesis during the BrdU pulse was 12 ± 1% (Fig. 5). This proportion was increased to 27 ± 1%, 38 ± 1% and 42 ± 7% by addition of 10 ng · ml^{-1}, 100 ng · ml^{-1} and 200 ng · ml^{-1} PDGF BB, respectively (Fig. 5). In response to 10%FCS the percentage of these cells in DNA synthesis to increased to 73 ± 3%. Taken together these results show that expression of the full- length human PDGFβ receptor did not effect PDGF BB induced DNA synthesis in rat VSMCs but cells expressing a truncated human PDGFβ receptor were unable to synthesize DNA in response to PDGF BB.

The cell line expressing the truncated PDGFβ receptor, described above, grew with the normal spindle shaped morphology of passaged VSMCs and all were grown under the same conditions. However, this cell line is derived from a selected population of cells and it is possible that the selection generated a cell line with a reduced dependence on and/or ability to respond to PDGF BB independent of truncated receptor expression. A second cell line expressing the truncated human PDGFβ receptor was therefore generated and the DNA synthesis response of this cell line to PDGF BB was analysed by BrdU incorporation. These cells showed a small increase in the proportion of nuclei synthesizing DNA in response to PDGF BB from 15 ± 2% in the absence of mitogen to 21 ± 2% and 24 ± 1% in the presence of 10 ng · ml^{-1} and 100 ng · ml^{-1} PDGF BB, respectively (Fig. 5). However, the increase in the proportion of nuclei synthesizing DNA was inhibited by 60% in the presence of 10 ng · ml^{-1} PDGF BB and 69% in the presence of 100 ng · ml^{-1} PDGF BB when compared with cells not expressing the truncated receptor (n = 4 counts of BrdU stained nuclei from each of 2 separate experiments). The maximum response to PDGF BB in this cell line was reached by the addition of between 10 and 50 ng · ml^{-1} suggesting that again the mechanism was a reduction in the total available active PDGFβ receptors by heterodimer formation. The difference between the results obtained from the two different cell lines expressing the truncated human PDGFβ receptor is likely to be due to a lower ratio of the truncated human PDGFβ receptor to the wild type rat PDGFβ receptor in the second cell line resulting in some residual signalling, however, this possibility has not been investigated.

In conclusion, the data presented in this study demonstrate that rat aortic VSMCs will correctly process and target to the cell membrane the protein for the human PDGFβ receptor and a truncated form of this gene. Expression of the full-length human gene had no effect on the response to PDGF BB of the cells whereas expression of the truncated PDGFβ receptor gene occurred at a level sufficient to block or inhibit the cells from entering DNA synthesis assayed by BrdU incorporation.

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