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Inactivation of platelet-derived growth factor-BB following modification by ADP-ribosyltransferase

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1 Arginine-specific ADP-ribosyltransferase (ART1) is expressed on the surface of a number of cell types, and catalyses the transfer of ADP-ribose from NAD^+ to target proteins. We investigated whether extracellular proteins such as growth factors may serve as substrates for this enzyme, with subsequent alteration in their biological activity. Experiments were performed with rat skeletal muscle membranes and V79 Chinese hamster lung fibroblasts with doxycycline-inducible expression of human ART.

2 From a panel of growth factors, platelet-derived growth factor-BB (PDGF-BB) was found to be the best substrate for ART1, whereas the structural homologue PDGF-AA was not a substrate. Under conditions of maximum labelling 5 mol ADP-ribose was incorporated per mol of PDGF-BB.

3 Purified (ADP-ribosyl)-PDGF-BB did not stimulate a mitogenic or chemotactic response in human pulmonary smooth muscle cells, and showed a reduced capacity to bind to PDGF receptors in competition binding experiments, when compared to unmodified PDGF-BB.

4 PDGF-dependent [^{3}H-methyl]-thymidine incorporation was measured in the ART1-transfected fibroblast cell line at physiological concentrations of PDGF-BB, and without addition of extracellular NAD^+. Fibroblasts expressing human ART1 at the cell surface showed reduced mitogenic responses to PDGF-BB, but not to PDGF-AA. This loss of mitogenic response in cells expressing ART1 activity was reversed by the addition of agmatine (an ART1 substrate).

5 In conclusion, we propose that PDGF-BB-dependent signalling may be regulated by post-translational modification of the growth factor by ART1 at the cell surface. This has been demonstrated in membranes of rat skeletal muscle, and the reaction confirmed in ART1-transfected fibroblasts.

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Abbreviations: ART1, Glycosylphosphatidylinositol-linked cell-surface arginine-specific ADP-ribosyltransferase; FBS, foetal bovine serum; (PA)SMC, (pulmonary artery) smooth muscle cell; PDGF, platelet-derived growth factor

Introduction

Platelet-derived growth factor (PDGF) is the most important mitogen of mesenchymal cells (Ross *et al*., 1986), which divide rapidly during embryogenesis, tissue remodelling and wound repair. PDGF comprises homo- or hetero-dimers of PDGF-A or PDGF-B, and the AA, AB and BB isoforms are all mitogenic in fibroblasts (Heldin *et al*., 1988; Seifert *et al*., 1989). Fibroblasts express α and β receptors, both of which bind PDGF-BB. Upon binding, the receptor subunits assemble as dimers and are autophosphorylated. This is the key event for subsequent intracellular signalling events and induction of mitogenic responses (Claesson-Welsh, 1994; Bornfeldt *et al*., 1995; Heldin, 1995). Cellular responses to PDGF have been shown to be regulated by variation in the abundance of cell-surface receptors (Barrett *et al*., 1996). However, PDGF receptor signalling may also be altered by the extracellular environment (Lin & Grinnell, 1993), and the possibility exists also that post-translational modification of PDGF may modify PDGF-dependent responses.

ADP-ribosyltransferase (ART1) serves a role in the post-translational modification of proteins by catalysing the transfer of an ADP-ribose moiety from NAD^+ to specific arginine residues of the target protein (Okazaki & Moss, 1996). ART1 is anchored to the outer aspect of the plasma membrane by a glycosylphosphatidylinositol side chain, and is found most abundantly in skeletal and heart muscle (Okazaki *et al*., 1994), and at lower levels in white blood cells (Wang *et al*., 1994; Kefalas *et al*., 1997) and airway epithelial cells (Balducci *et al*., 1999). Similar enzyme activity has also been detected in endothelium (Jones & Baird, 1997). The catalytic activity of ART1 is similar to that of cholera toxin, although its primary structure and protein substrates are different. The protein substrates of ART1 identified to date are mainly located on the cell surface, and include the external domains of integrin subunits α7 (Zolkiewska &

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Moss, 1993), αL (CD11a) and β2 (CD18) (Nemoto *et al*., 1996), and a 40 kDa protein which modifies tyrosine kinase activity of $p56^{lck}$ (Wang *et al*., 1996).

Basic fibroblast growth factor (FGF) has also been identified as an ART1 substrate (Jones & Baird, 1997). The consequences of the ADP-ribosylation of FGF have yet to be fully characterized, but the possibility has been considered that they might be associated with altered binding affinity or reduced capacity to transmit the intracellular signals that follow binding of FGF to its receptor. There are no previous reports of ART1-dependent modification of PDGF, but the central role of PDGF in growth and differentiation prompted us to consider such a reaction.

Numerous reactions between NAD^+ and proteins have been reported, and the transfer of ^{32}P from [$\alpha^{32}P$]-NAD^+ to a protein cannot be taken (on its own) as evidence of ART1 catalytic activity. NAD^+, for example, forms NAD-protein adducts spontaneously, and the reaction is facilitated by NO donors (Boyd *et al*., 1993; McDonald & Moss, 1993). To eliminate these confounding effects in the present studies, V79mz Chinese hamster lung fibroblasts, which are responsive to PDGF, were transfected with human ART1 cDNA (Yadollahi-Farsani *et al*., 1999). The stable expression of ART1 in these cells is under the control of a tetracycline-sensitive promoter, and they provide a robust system with which to examine the effect of ART1 expression on PDGF-dependent signalling. The results show that (i) PDGF-BB is a substrate for ART1, (ii) the reaction is accompanied by reduced PDGF-BB-dependent responses, (iii) that the modification of the growth factor occurs within the microenvironment of the cell surface.

Methods

Cell culture

Unless otherwise stated, all cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. Human pulmonary artery smooth muscle cells (PASMC) (a kind gift from Dr N. Morrell, Cambridge, U.K.) were derived from pulmonary artery of resected lung specimens from donor tissue for lung transplantation (Morrell *et al*., 1999). Cells were cultured in M199 medium supplemented with 10% FBS, 2 mM L-glutamine, 5 u ml^{-1} penicillin and 5 u ml^{-1} streptomycin. Cells were used in experiments between passages 3 and 10.

Chinese hamster lung fibroblasts (V79mz) were cultured in monolayers in DMEM supplemented with 10% foetal bovine serum and 2 mM L-glutamine. A cell line (V79-ART1on), with doxycycline-inducible expression of human ART1 was established using the Tet-On mammalian gene expression system (Clontech, CA, U.S.A.) (Yadollahi-Farsani *et al*., 1999). Stably transfected cells were maintained in selective medium containing 200 μg ml^{-1} geneticin and 100 μg ml^{-1} hygromycin, and maximum expression of cell surface ART1 was induced by addition of 2 μg ml^{-1} doxycycline to the medium for 48 h.

Rat skeletal muscle membrane preparation

Rat thigh muscle was homogenized on ice in 50 mM Tris HCl buffer, pH 7.4, containing 0.25 mM EDTA and 290 mM sucrose. Cell debris was removed by centrifugation at $200 \times g$ for 15 min. Membranes were pelleted from the supernatant by ultra-centrifugation at $40{,}000 \times g$, for 1 h. The membranes were resuspended in 50 mM Tris HCl buffer pH 7.4, containing 0.25 mM EDTA, and the protein concentration was estimated using the BCA assay (Pierce & Warriner, Chester, U.K.). In subsequent ADP-ribosylation experiments, 100 μg of membrane protein was used.

ADP-ribosylation of growth factor

V79-ART1on cells were seeded in 24-well culture plates at 5×10^4 per well, and cultured for 48 h in medium containing 2 μg ml^{-1} doxycycline. The cells were washed twice with serum free medium, and then incubated with 100 μl DMEM containing 25 mM HEPES buffer pH 7.4, 0.1% BSA, 10 μM NAD^+, 10 μCi [α-^{32}P]-NAD (Nycomed Amersham, Little Chalfont, U.K.), 1 mM ADP-ribose and 0.1–1 μg of recombinant human PDGF (Sigma, Poole, U.K.) for the times indicated. Growth factor was resolved on 15% SDS–PAGE, blotted on to nitrocellulose, and ADP-ribosylated growth factors were identified by autoradiography.

Immunoprecipitation and Detection of PDGF and (ADP-ribosyl)-PDGF

The ADP-ribosylation reaction was terminated by transfer onto ice, with removal of cells or membranes by centrifugation at $15{,}000 \times g$. The reaction mix was diluted with 0.5 ml PBS containing 1% (v v^{-1}) NP-40, 1 mM thymidine monophosphate nitrophenylester and 10 mM NAD^+. Non-specific binding to IgG was minimized by pre-clearing with 10 μg normal goat IgG for 1 h at 4°C, and precipitation with protein G-agarose (Amersham Pharmacia, Chalfont, U.K.). PDGF was immunoprecipitated using 10 μg goat anti-PDGF purified IgG antibody (Sigma, Poole, U.K.) raised against recombinant PDGF, and protein-G-agarose. Immunoprecipitates were resolved on 15% SDS–PAGE, blotted on to nitrocellulose, and [^{32}P]-ADP-ribosyl-PDGF identified by autoradiography. Following autoradiography, the position of authentic PDGF-B was confirmed by Western blotting using anti-C-terminal peptide of PDGF-B (Santa Cruz Biotechnology, California, U.S.A.) with ECL detection.

Purification of (ADP-ribosyl)-PDGF-BB

For these experiments, the conditions were modified to maximize the ADP-ribosylation of PDGF-BB. ART1 was released from the surface of V79-ART1on cells with phosphoinositide-specific phospholipase C as described previously (Kefalas *et al*., 1999). Reactions of 100 μl contained 20 mM HEPES buffer pH 7.4, 1 μg PDGF-BB, 200 μM NAD^+ (10 μCi [^{32}P]-NAD^+), 1 mM ADP-ribose, 1 mM thymidine monophosphate nitrophenylester, 0.11 μg aprotinin, 0.1 μg leupeptin, 0.1 mM PMSF, 0.1 mM EDTA and ART1 enzyme from 1×10^6 cells (approx.). The reaction was allowed to proceed for 17 h at 37°C.

(ADP-ribosyl)-PDGF-BB was purified from the reaction mix using a Vydac C-4 reverse phase HPLC column (i.d. 4.6×150 mm) (HighChrom Ltd, Reading, U.K.). (ADP-

ribosyl)-PDGF-BB was separated from NAD^+ and unmodified PDGF-BB with a gradient of 10–40% acetonitrile in 0.1% (v v^{-1}) trifluoroacetic acid at a rate of 1 ml min^{-1} over 30 min. ^{125}I-PDGF-BB (50,000 c.p.m.) was added to the reaction mix as tracer. The concentration of purified PDGF and incorporation of ADP-ribose was determined by measuring the dual labels ^{125}I and ^{32}P respectively by scintillation counting (Packard Tricarb 2100).

Migration

Confluent PASMC were harvested with trypsin, washed three times in medium and resuspended at 1 million cells ml^{-1} in medium containing 10% FBS and 20 mM HEPES buffer pH 7.4. 50,000 cells were added to the upper chamber of a modified micro-Boyden chamber and the number of cells migrated across an 8 μm pore polycarbonate filter was determined as described previously (Saxty *et al*., 1998).

Radioligand binding

PASMC cells were seeded at 20,000 cells $well^{-1}$ on 24-well plates, grown to 90% confluency and serum starved for 24 h. The cells were washed twice with binding buffer (M199 supplemented with 1% BSA, 20 mM HEPES buffer pH 7.4) and cooled on ice. The cells were incubated at 4°C in binding buffer with shaking for 2 h in the presence of 50,000 c.p.m. ^{125}I-PDGF-BB (80 pM) (NEN, Hounslow, U.K.) plus PDGF-BB or (ADP-ribosyl)-PDGF-BB at the concentrations shown. The cells were washed rapidly five times in ice cold PBS containing 1% BSA, and ^{125}I-PDGF-BB bound to the cells was solubilized in 1% Triton X-100 in water, containing 1% BSA, and counted in a scintillation counter. Non-specific binding was determined in the presence of an excess of unlabelled PDGF-BB (16 nM), and subtracted from all values. The data were fitted to a simple one-site binding model, and IC_{50} values were determined using Graphpad Prism software.

[^{3}H-methyl]-thymidine incorporation

[^{3}H-methyl]-thymidine incorporation was measured by a modification of the method of Bowen-Pope (1982). PASMCs were seeded in 24-well plates at a density of 20,000 $well^{-1}$, grown to 80–90% confluence, then were growth arrested by serum starvation for 2 h, followed by exposure to M199/0.1% FBS for 72 h. The medium was then exchanged for fresh M199/0.1% FBS containing growth factor and 0.25 μCi/well [methyl-^{3}H]-thymidine (Nycomed Amersham, Little Chalfont, U.K.), and the cells were cultured for a further 24 h. The cells were washed thoroughly in ice-cold phosphate buffered saline, and trichloroacetic acid (10%) insoluble counts were measured by scintillation counting. V79-ART1on cells were treated as described above with the following minor changes. Throughout the experiment, V79-ART1on cells were cultured in the absence or presence of 2 μg ml^{-1} doxycycline. Fresh medium containing 0.1% BSA and growth factor was added to the growth-arrested cells, and incubated for 18 h, followed by 1 h pulse labelling with [^{3}H-methyl]-thymidine (0.4 μCi $well^{-1}$).

Results

ADP-Ribosylation of PDGF-BB

The capacity of PDGF-BB to serve as a substrate for ART1 was investigated using rat skeletal muscle membrane preparations. Figure 1 shows ADP-ribosylated PDGF-BB which has been immunoprecipitated from the membrane and resolved on 15% SDS–PAGE. The same reaction was also demonstrated using intact V79-ART1on cells, in which modification of PDGF-BB was dependent on the expression of ART1, with no labelling observed in cells that had not been exposed to doxycycline. In each case, the presence of authentic PDGF-BB in the immunoprecipitate was confirmed by Western blotting with ECL detection. The position of ^{32}P-labelled protein and immunoprecipitated PDGF were found to be coincident. It was also confirmed that the ^{32}P-labelled product was sensitive to treatment with neutral hydroxylamine or alkaline conditions (data not shown), which identified the labelled product as (ADP-ribosyl)-arginine within PDGF-BB (Cervantes-Laurean *et al*., 1993).

The selectivity of the incorporation of the [^{32}P]-label from [^{32}P]-NAD to a panel of growth factors, catalysed by cell surface ART1 was investigated and is shown in Figure 2a. Strong incorporation was observed into fibroblast growth factor, insulin-like growth factor and PDGF-BB. PDGF-AA (a structurally similar molecule, sharing 70% amino acid homology with PDGF-BB) was not a substrate for the enzyme. The generation of (ADP-ribosyl)-PDGF-BB increased with time (Figure 3a) and PDGF-BB concentration (Figure 3b). The purification of (ADP-ribosyl)-PDGF-BB involved reverse-phase h.p.l.c, which is shown in Figure 4a. Baseline separation of (ADP-ribosyl)-PDGF-BB and authentic PDGF-BB (using ^{125}I-PDGF-BB tracer) was achieved, and it was confirmed on non-reducing SDS–PAGE that the ADP-ribosylated form of PDGF was dimeric (Figure 4b). Under conditions of maximum labelling with [^{32}P]-NAD^+, the stoichiometry of ADP-ribose:PDGF-BB dimer was

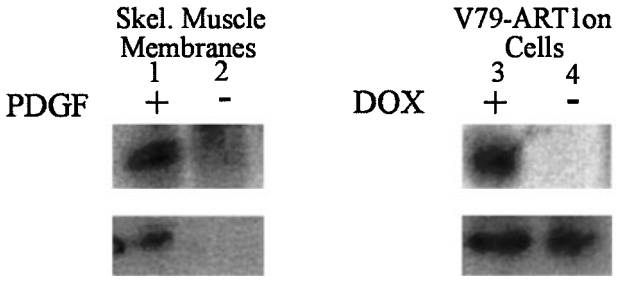

Figure 1 ADP-ribosylation of PDGF-BB. Skeletal muscle membranes or V79-ART1on cells were incubated with [^{32}P]-NAD and with (lanes 1, 3 and 4) or without (lane 2) 0.5 μg PDGF-BB as described in Methods. PDGF-BB was immunoprecipitated in each case and resolved on 15% SDS–PAGE and blotted on to nitrocellulose. The blot was exposed to X-ray film, and subsequently probed with an anti-PDGF-B antibody (with ECL development). The upper panels show autoradiographs of the 6–16 kDa region of the blots, and the lower panels the corresponding Western blots. Lanes 1 and 2 show PDGF-B immunoprecipitates from reactions with skeletal muscle membranes in the presence or absence of PDGF-BB. Lanes 3 and 4 show PDGF-B immunoprecipitates from reactions with V79-ART1on cells, cultured in the presence (lane 3) or absence (lane 4) of doxycycline.

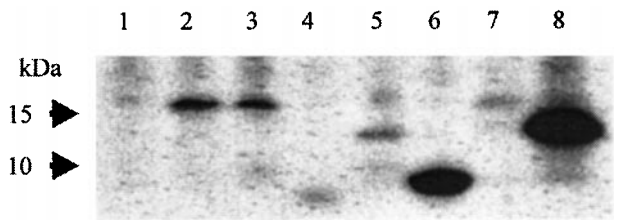

Figure 2 ADP-ribosylation of growth factor. (a) V79-ARTon cells expressing ART1 at the cell surface were incubated with 1 μg of each growth factor and [^{32}P]-NAD$^+$ as described in Methods. The [^{32}P]-labelled growth factors were resolved by SDS–PAGE, blotted on to nitrocellulose and detected by autoradiography. Lane 1, none; lane 2, basic fibroblast growth factor (16.4 kDa); lane 3, acidic fibroblast growth factor (15.5 kDa); lane 4, epidermal growth factor (6 kDa); lane 5 heparin binding epidermal growth factor (9.5 kDa); lane 6, insulin-like growth factor (7.5 kDa); lane 7, platelet-derived growth factor-A (16 kDa); lane 8, platelet-derived growth factor-B (14 kDa).

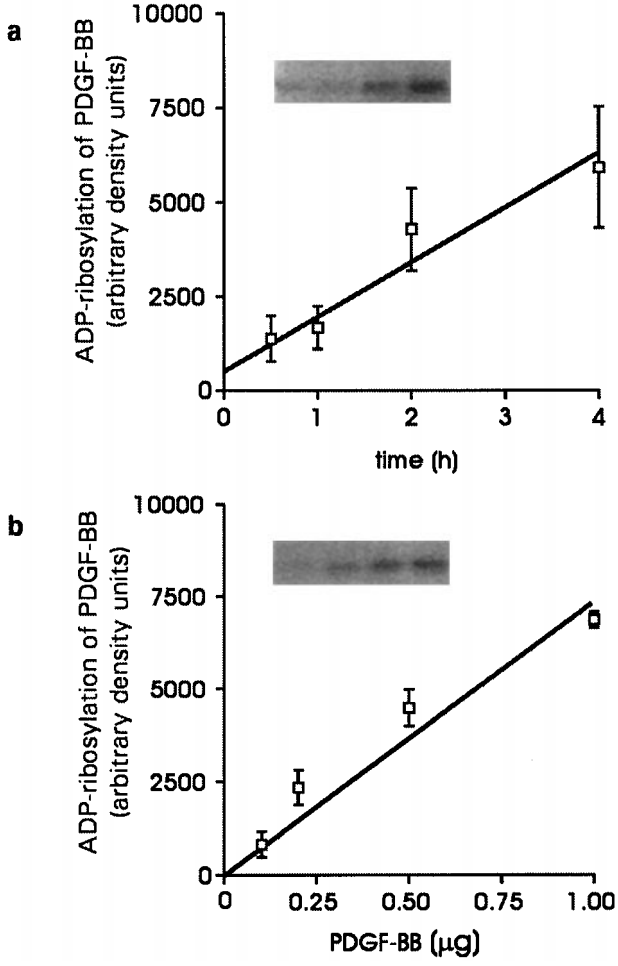

Figure 3 ADP-ribosylation of PDGF-BB. The incorporation of [^{32}P]-ADPribose into PDGF was determined by densitometric scanning of autoradiographs (shown in insert). (a) Time course for the generation of (ADP-ribosyl)-PDGF-BB by ART1 expressed on V79-ART1 on cells. (b) (ADP-ribosyl)-PDGF-BB generation by ART1 expressed on V79-ART1on cells in the presence of selected concentrations of PDGF-BB.

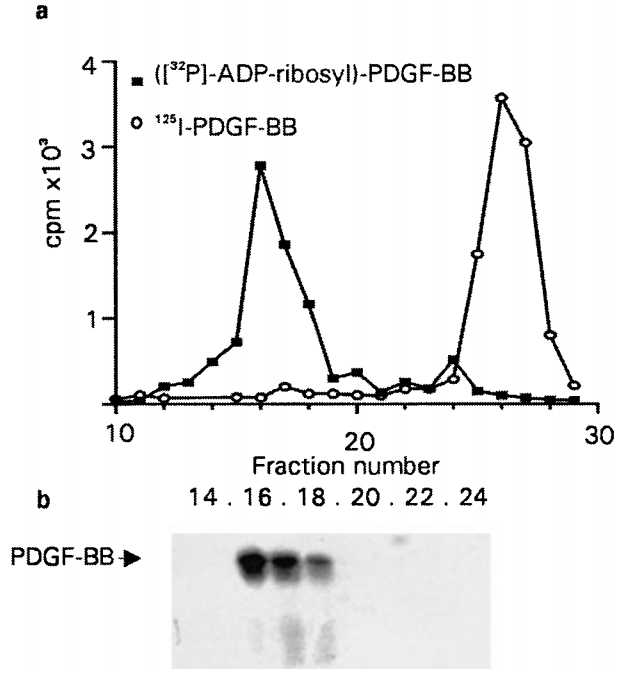

Figure 4 Purification of (ADP-ribosyl)-PDGF-BB. (ADP-ribosyl)-PDGF-BB was generated by exposure of V79-ART1on cells to ^{32}P-NAD$^+$ and PDGF-BB, and was purified on a C4 reverse-phase HPLC column as described in Methods. (a) The elution patterns of authentic PDGF-BB using ^{125}I-PDGF-BB as a tracer and ([^{32}P]-ADP-ribosyl)PDGF-BB from two separate runs are shown. (b) The corresponding fractions containing ([^{32}P]-ADP-ribosyl)-PDGF-BB were analysed on 12.5% SDS–PAGE under non-reducing conditions, followed by blotting on to nitrocellulose and autoradiography. The relative mobility of authentic (dimeric) PDGF-BB is indicated on the left.

estimated to be 5:1, from the ratio of the [^{32}P] radiolabel associated with (ADP-ribosyl)-PDGF, and the [I^{125}]-PDGF tracer used in the reaction (see Methods). The bacterial ADP-ribosyltransferases modify their eukaryotic protein substrates by the addition of ADP-ribose to single amino acid residues, and the modification of integrin α7 by mouse ART1 was also restricted to a single arginine residue (Zolkiewska & Moss, 1993). The present demonstration of multiple acceptor sites on PDGF-BB under conditions of maximum labelling is an unexpected observation, and further studies will be required to identify which of the modified arginine residues leads to inactivation of the growth factor.

ADP-ribosylation of PDGF-BB alters biological activity

The biological activity of (ADP-ribosyl)-PDGF-BB was determined by measuring the chemotactic and proliferative responses of human pulmonary artery smooth muscle cells. Table 1 shows the responses observed with 400 pM unmodified PDGF-BB or (ADP-ribosyl)-PDGF-BB, or foetal bovine serum control. PDGF-BB induced a 2.5 fold increase in the number of PASMC cells migrating across a polycarbonate filter, and a 9 fold increase in proliferation of growth-arrested cells compared to serum controls. Examination of the data for (ADP-ribosyl)-PDGF-BB showed that the chemotactic and proliferative responses were not significantly different from serum controls.

Table 1 Comparison of the biological activities of (ADP-ribosyl)-PDGF-BB and PDGF-BB

	FBS control	*ADPribosyl-PDGF-BB* (10 ng ml^{-1})	*PDGF-BB* (10 ng ml^{-1})
Chemotaxis (cells migrated)	36±16	30±10	76±16*
Proliferation (TCA-insoluble c.p.m./well)	5728±554	6126±723	52055±1499*

The chemotactic and mitogenic responses of PDGF-BB and (ADP-ribosyl)-PDGF-BB were tested in cultured human pulmonary artery SMC. *Chemotaxis*: The number of cells that migrated to the lower surface of a polycarbonate filter in a 180 μm^2 field ($n=9$) in response to added growth factor was compared to 10% FBS control. *Proliferation*: [^{3}H-methyl]-thymidine counts incorporated into growth-arrested cells in response to added growth factor were compared to 0.1% FBS control. *Indicates mean was significantly different ($P<0.01$) from control using two tailed, Student's *t*-test.

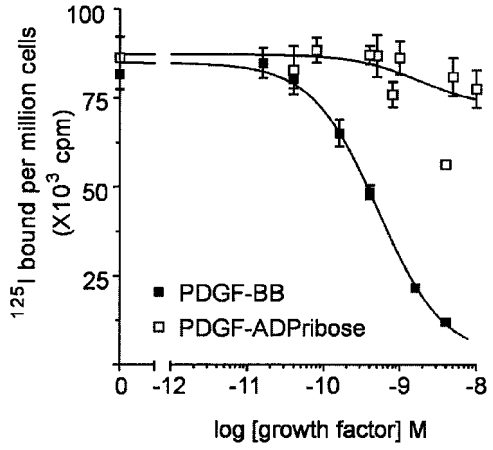

Figure 5 PDGF receptor binding. The results show ^{125}I-PDGF-BB (80 pM) binding to intact human pulmonary artery SMC cells, and inhibition of the binding by selected concentrations of PDGF-BB or (ADP-ribosyl)PDGF-BB. Data are shown as mean values±s.e.mean from three separate experiments.

The reduced biological activity of (ADP-ribosyl)-PDGF-BB when compared to PDGF-BB was confirmed in competition binding experiments with ^{125}I-PDGF-BB. In Figure 5, it can be seen that the binding affinity of (ADP-ribosyl)-PDGF-BB ($IC_{50}>10$ nM) was greatly reduced compared to authentic PDGF-BB ($IC_{50}=350-758$ pM; 95% confidence interval). Binding experiments were also performed with the hamster V79 fibroblast cell line, in which similar results were observed, $IC_{50}>15$ nM compared to an IC_{50} of 400–650 pM for binding of authentic PDGF-BB.

Expression of ART1 inhibits the PDGF-BB-dependent mitogenic response

To investigate whether PDGF-BB was modified by ART1 under physiological conditions, i.e. picomolar concentrations of PDGF-BB, in the absence of added extracellular NAD, a comparison was made of the mitogenic effect of PDGF on V79-ART1on cells. In these cells, the expression of ART1 at the cell surface was controlled by the addition of doxycycline. Concentration-response curves for PDGF are presented in Figure 6, which shows the incorporation of [^{3}H-methyl]-thymidine into growth arrested cells cultured in the absence (control) or presence of doxycycline (ART1 expressing), and on untransfected V79mz controls.

ART1-expressing V79-ART1on cells showed a 50% reduction in the maximal mitogenic response to PDGF-BB compared to control V79-ART1on cells (Figure 5a). Control V79-ART1on cells (cultured in the absence of doxycycline, Figure 5a) showed similar PDGF-BB dependent mitogenic responses to the untransfected V79mz cells (cultured in the absence or presence of doxycycline; Figure 5b). This confirmed that the mitogenic response in Figure 5a was not reduced as a consequence of any cytotoxic effect of doxycycline. The stimulation of [^{3}H-methyl]-thymidine incorporation into V79-ART1on cells by PDGF-AA was not altered by the expression of ART1 (Figure 5c), and was similar to the untransfected control (Figure 5d). This was consistent with the earlier observation that PDGF-AA was not a substrate for ART1.

Further evidence in support of the proposal that ADP-ribosylation of PDGF-BB is followed by inactivation of the growth factor was obtained using agmatine (decarboxy-arginine), a low molecular weight substrate of ART1 (Moss & Vaughan, 1984), which serves as a competitive inhibitor of the ADP-ribosylation of proteins. [^{3}H-methyl]-thymidine incorporation into growth arrested V79-ART1on cells cultured in the absence (control) or presence of doxycycline (ART1 expressing) in response to 10 ng ml^{-1} PDGF-BB was measured as described in Methods. Measurements were then made in the absence or presence of 10 mM agmatine, which was added with the PDGF-BB. In the absence of agmatine, ART1-expressing cells incorporated 4730±815 c.p.m. [^{3}H-methyl]-thymidine in response to PDGF compared to 8970±980 c.p.m. in ART1-non-expressing cells. In the presence of 10 mM agmatine both ART1 expressing and non-expressing cells showed similar incorporation of [^{3}H-methyl]-thymidine (8000±1100 and 8640±490 respectively).

Discussion

The results presented confirm a previous report (Jones & Baird, 1997) that extracellular proteins, such as growth factors are substrates for ART1. Both PDGF-BB and fibroblast growth factor-2 (FGF-2) are soluble, arginine-rich, low molecular weight proteins, which are ADP-ribosylated when exposed to exogenous NAD^+ in the presence of ART1. However, there was striking substrate specificity in this reaction. PDGF-AA is structurally homologous to PDGF-BB and shares many conserved arginine residues (Oefner *et al*., 1992), however PDGF-AA was not a substrate for the enzyme.

In adults, ART1 is most abundantly expressed in skeletal muscle tissue (Zolkiewska & Moss, 1993), and we were able to demonstrate labelling of PDGF-BB from membrane preparations of this tissue. The physiological significance of the expression of ART1 in myocytes is not known, although inhibitors of ADP-ribosylation have been shown to block differentiation and proliferation of myocytes during myogen-

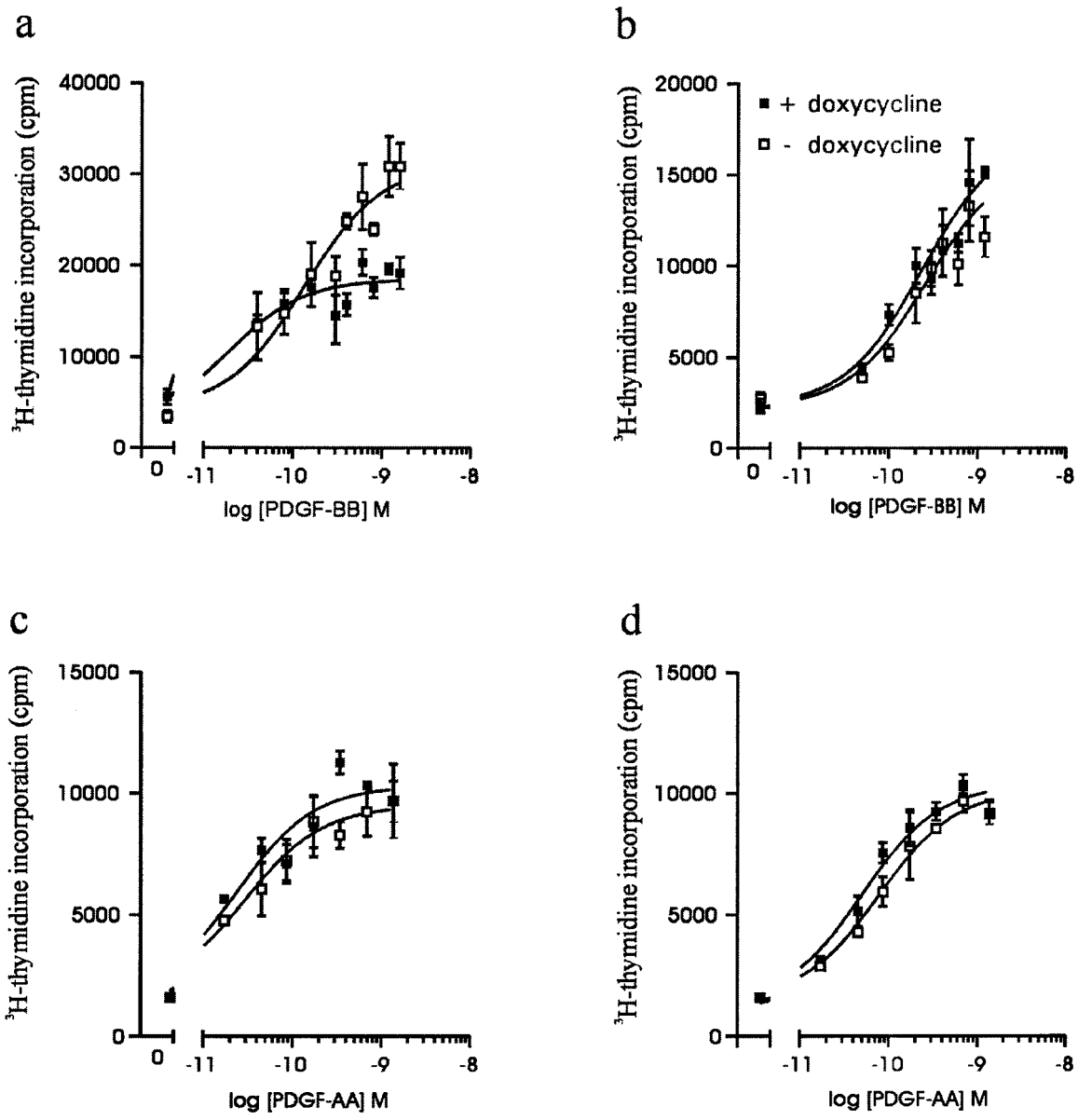

Figure 6 [^{3}H-methyl]-thymidine incorporation. The results are shown of [^{3}H-methyl]-thymidine incorporation into cells cultured in the absence or presence of doxycycline (ART1 expressing), and are plotted as the means$\pm$s.e.mean, $n=9$: (a) and (c) the mitogenic response of V79-ART1on cells following exposure to selected concentrations of PDGF-BB or PDGF-AA respectively; (b) and (d) the mitogenic response of untransfected V79mz cells in the presence and absence of doxycycline following exposure to selected concentrations of PDGF-BB or PDGF-AA respectively.

esis (Kharadia *et al*., 1992). The role of PDGF-BB in these processes has been reported previously (Jin *et al*., 1993).

The identity of PDGF as a substrate for ART1 was confirmed in a transfected cell line in which the expression of the enzyme was under the control of a tetracycline sensitive promoter. Purification of (ADP-ribosyl)-PDGF-BB demonstrated that the modification was associated with reduced signalling capacity in smooth muscle cells, and reduced binding affinity for PDGF-receptor. These results suggested that cell responsiveness to PDGF-BB may be altered by expression of ART1 at the cell surface. Such a mechanism could have an important role in development, wound healing and haemostasis. There are examples in an *in vitro* model of wound healing, where a lack of PDGF-BB signalling has been observed without changes in receptor concentration (Lin & Grinnell, 1993).

To address this possibility, in the absence of specific inhibitors or antibody, we utilized an ART1 transfected cell, in which expression of the enzyme was induced by doxycycline. In these experiments physiologically relevant concentrations of PDGF-BB were added to cultured cells in the absence of exogenous NAD^+. Expression of ART1 at the cell surface attenuated [^{3}H-methyl]-thymidine uptake into dividing cells, which was consistent with inhibition of PDGF-BB binding to its receptor. Furthermore, the experiment confirmed that the modification of PDGF-BB was rapid enough to exert a measurable effect within the time course of the experiment.

Previous experiments have shown that incubation of V79-ART1on cells with [^{32}P]-NAD$^+$ results in the modification of a number of cell-surface proteins (Yadollahi-Farsani *et al*., 1999). To exclude the possibility that ADP-ribosylation of the PDGF receptor itself accounted for the reduction in PDGF-BB-dependent responses observed, a comparison was made of the mobility on polyacrylamide gels of [^{32}P]-ADP-ribosylated membrane proteins extracted from ART1-expressing V79-ART1on cells and the 180 kDa PDGF receptor. No incorporation of the ^{32}P-label into the PDGF receptor was observed (data not shown). The possibility is not totally excluded, however, that another protein on the cell surface was also ADP-ribosylated, which then altered receptor signalling, perhaps by a mechanism similar to that proposed by Wang *et al*. (1996). Fibroblasts express both α and β PDGF-receptors which bind the PDGF-BB isoform. PDGF-AA binds to the α receptor only, and its mitogenic activity was not altered by expression of ART1 at the surface. This was consistent with the observation that PDGF-AA was not a substrate for ART1, and suggested also that downstream signalling events in mitogenesis were unaffected by expression of the enzyme.

In conclusion we report a novel mechanism for the regulation of PDGF-BB activity at the cell surface, involving ART1-dependent transfer of ADP-ribose from NAD$^+$ to arginine residues of the growth factor. In the present experiments (see Figure 6), ART1 was competent to utilize endogenous NAD$^+$ (since none was added). ART1 is one of a family of NAD$^+$-dependent enzymes located on the cell surface, which include cyclic ADP-ribose synthase (Lee, 1996) and NAD$^+$-glycohydrolase (Kim *et al*., 1993; Aleo *et al*., 1996). The transporter involved in the export of NAD$^+$ from the cytosol to its extracellular location has never been identified however. ART1 is widely distributed in myocytes, endothelial cells and haematopoietic tissues (Wang *et al*., 1994; Kefalas *et al*., 1997; Jones & Baird, 1997), and we propose that it may serve a physiological role to regulate the mitogenic activity of PDGF-BB in these tissues.

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