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Endogenously elicited antibodies to platelet derived growth factor-BB and platelet cytosolic protein inhibit aortic lesion development in the cholesterol-fed rabbit

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Summary. Several studies have indicated that growth factors, such as platelet derived growth factor (PDGF), may be important in atherogenesis. These factors are released from platelets, or expressed by cells of the arterial wall. In order to study their role in atherogenesis more directly, rabbits were immunized with PDGF-BB, platelet cytosolic protein, or human serum albumin (HSA), until high titres of antibody were attained. Atherosclerotic lesions were subsequently induced by feeding the animals with a 2% cholesterol enriched diet. At the end of approximately 3 months, the extent of aortic lesion development was assessed by image analysis of *en face* preparations of aortae stained with Oil Red-O, and histological segments of aortae taken at the level of the first intercostal artery branch point. The endogenous antibodies were characterized with respect to their cross-reactivity, and ability to neutralize PDGF and platelet cytosol-induced cell proliferation and migration *in vitro*. The endogenous, anti-PDGF-BB antibody was isoform specific, and neutralized the mitogenic and chemotactic properties of PDGF-BB and rabbit platelet cytosolic protein *in vitro*. The anti-platelet cytosol antibody partially inhibited the chemotactic and mitogenic properties of rabbit platelet cytosolic protein. Compared to non-immune rabbits ($n = 5$), animals immunized with HSA ($n = 4$) had a significantly larger area of aortic lesion involvement ($P < 0.01$), whereas aortic lesions in rabbits immunized with PDGF-BB ($n = 5$), or platelet cytosolic protein ($n = 7$) were significantly smaller than either non-immune animals, or animals immunized with HSA ($P < 0.05$). The same pattern was observed for other measures of aortic lesion involvement including aortic intima : media ratio at the level of the first intercostal artery. These data suggest that PDGF-BB, and possibly other platelet-associated growth factors, are involved in cholesterol-induced atherosclerosis.

Keywords: rabbit, cholesterol diet, PDGF-BB, platelet cytosolic protein, endogenous antibodies, aortic lesions

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The fatty-streak is the earliest macroscopically evident lesion of atherosclerosis. This lesion is predominantly composed of macrophage-derived foam-cells, which accumulate beneath an intact endothelial cell layer. Subsequent smooth muscle cell migration and proliferation appear to be key events in lesion progression, leading to the formation of the fibro-fatty plaque (reviewed by Ross 1993). The factors responsible for these two processes are unknown, although recent work indicates that platelet derived growth factor (PDGF) may play an important role in atherogenesis (Ross *et al*. 1990).

PDGF is a cationic protein with a molecular weight of approximately 30 kDa (reviewed by Ross *et al*. 1986). The active PDGF molecule is dimeric, consisting of one of three combinations (PDGF-AA, AB and BB) of two homologous polypeptide chains, termed PDGF-A and B, which display marked cross-species homology (Wang *et al*. 1992). PDGF and its receptor are both expressed by cells involved in the atherogenic process (Raines *et al*. 1990). PDGF is additionally present within platelets, and is released following their degranulation. Platelet associated proteins, including PDGF-BB, enhance the uptake and degradation of modified LDL, and cholesterol accumulation by macrophages (Fuhrman *et al*. 1992, also reviewed by Aviram 1995), processes that are likely to be critical in atherosclerotic lesion expansion (Steinberg *et al*. 1989). Barrett and Benditt (1988) have reported that PDGF-B chain expression is enhanced in atherosclerotic lesions and that this is associated with increased expression of c-fms, a marker for the presence of macrophages. Although studies by Wilcox *et al*. (1988) using *in situ* hybridization could not demonstrate any evidence of enhanced PDGF gene expression, Ross and colleagues (1990) have shown more recently that PDGF-B chain gene and protein expression are increased in experimentally induced atherosclerotic lesions in monkeys and advanced lesions in man. They report that the PDGF-B chain is primarily expressed by macrophages and is associated with enhanced IL-1β expression, an indication of macrophage activation. The discrepancy between these studies has been attributed to the type of pathological material used for analysis. The role of PDGF-AA in atherogenesis is less clear, although it is expressed by vascular smooth muscle cells, is present within human platelets and atherosclerotic lesions (Rehkter & Gordon 1994), and has been shown to mediate the mitogenic effect of other growth factors, including TGF-β and IL-1β, via autocrine loops (Battegay *et al*. 1990; Raines *et al*. 1989).

Cell proliferation and migration are probably driven by the synergistic interplay of several growth factors and cytokines (reviewed by Ross 1993). Initially, these factors are probably derived from activated leucocytes (macrophages, macrophage-derived foam-cells and T lymphocytes) within the lesion, and from dysfunctional endothelial cells (Katsuda *et al*. 1993; Bohm *et al*. 1994). Smooth muscle cells are also capable of secreting factors such as PDGF-AA, IGF-I and TGF-β which may act in an autocrine or paracrine manner. At a later stage, the endothelial lining may become more seriously compromised resulting in cell retraction or denudation. The exposure of thrombogenic elements of the extracellular matrix may then lead to platelet deposition and degranulation, causing the release of platelet-associated growth factors.

Coronary angioplasty, bypass grafting and cardiac transplantation may all be associated with rapidly developing recurrent disease, which is characterized by the formation of a concentric, occlusive, neo-intimal lesion. Smooth muscle cell migration and proliferation are thought to be responsible for this process (Clowes *et al*. 1983). We (Ferns *et al*. 1991; Rutherford *et al*. 1996) and others (Lindner & Reidy 1991; Jawien *et al*. 1992; Jackson & Reidy 1993; Jackson *et al*. 1993) have previously shown that PDGF and bFGF are at least partly responsible for the development of a neo-intima following balloon catheter induced injury in the rat. Further support for a possible role of PDGF-B chain in neo-intimal hyperplasia arises from experiments in which vascular endothelial cells were transfected with gene constructs for PDGF-B chain, and was associated with intimal thickening (Nabel *et al*. 1993; Pompili *et al*. 1995).

We hypothesized that PDGF-BB, derived from monocyte/ macrophages, platelets, or dysfunctional endothelial cells, is involved in early atherosclerotic lesion development, through its effects on macrophage and smooth muscle cell function. In order to test this hypothesis we used an active immunization approach in which rabbits were initially injected with PDGF-BB until high titres of neutralizing antibodies were elicited and atherosclerosis was subsequently induced by the institution of a high cholesterol diet.

Materials and methods

Rabbit colonies

Twenty-eight juvenile New Zealand White rabbits (3 months old), weighing approximately 2.5 kg, were housed in the Biological Services Unit of St Bartholomew's Hospital Medical College, London.

Immunizations

Recombinant PDGF-BB was purchased from Bachem UK Ltd (Suffolk), and human serum albumin from Sigma

Chemical Co. Ltd. Rabbit platelet cytosolic protein was prepared from citrated whole blood. Platelet-rich plasma was obtained by centrifuging at 200 ***g*** for 20 minutes. Prostacyclin (final concentration 0.33 μg/ml) (Sigma Chemical Co., Poole, UK) was added and the platelet-rich plasma centrifuged for a further 15 minutes at 800 ***g***. The platelet pellet was resuspended in phosphate buffered saline (pH 7.4), and the platelets lysed by five cycles of freeze-thawing. The released platelet proteins were separated by centrifugation at 10 000 ***g*** and the protein concentration in the supernatant measured by a Lowry protein assay (Lowry *et al*. 1951). Immunogens were administered by subcutaneous injection. The initial injection (75 μg of PDGF-BB ($n = 7$) or HSA ($n = 7$), or 2 mg platelet cytosolic protein ($n = 8$)) was in complete Freund's adjuvant given as four 0.25 ml injections at multiple sites. Subsequent injections (37.5 μg PDGF-BB, 37.5 μg HSA, or 1 mg platelet cytosolic protein) were given in incomplete Freund's. Non-immune rabbits were injected with Freund's adjuvant containing saline alone ($n = 6$). Antibody titres were measured using an ELISA as previously described (Ferns *et al*. 1991).

Antibody assessment

Plasma was collected from each animal at the time of sacrifice. The immunoglobulin fraction was partially purified by sodium sulphate precipitation as previously described (Rutherford *et al*. 1997), and the antibody assessed for its specificity and ability to neutralize the bioactivity of a fixed amount of PDGF, as previously described (Rutherford *et al*. 1997), by (i) Western blotting of a rabbit platelet lysate preparation, (ii) mitogenesis assay, using rabbit smooth muscle cells and 3T3-fibroblasts stimulated with either PDGF-AA, -AB or -BB, and (iii) rabbit smooth muscle cell chemotaxis assay using PDGF-BB.

Dietary groups

Once adequate antibody titres to PDGF-BB, platelet cytosolic protein, or HSA were elicited, the animals were started on a 2% cholesterol-enriched diet (Special Dietary Services, Essex, UK). Plasma cholesterol levels were measured at approximately 4-weekly intervals. Water was allowed *ad libitum*.

Blood sampling

Blood for serum cholesterol and plasma specific-IgG levels was collected from each animal before the start of each experimental diet, monthly thereafter and at the time of sacrifice. Serum or plasma was obtained by centrifugation and stored at -20°C prior to analysis.

Animal killing

Approximately 18 weeks after the initiation of the cholesterol enriched diet, the animals were sedated with intramuscular xylazine and ketamine. An abdominal incision was then made to access the abdominal aorta for insertion of a cannula connected to a perfusion apparatus, and both jugular veins exposed for perfusion run-off. The animals were given heparin (1000 iu/kg) 15 minutes before being killed by an overdose of anaesthetic (sodium pentobarbital). The animals were then perfused with buffered saline at a rate of 100 ml/minute/kg body weight. When the run-off was clear, the saline was replaced with 4% paraformaldehyde in isotonic PBS at the same flow rate. Perfusion was continued for 15 minutes. After fixation *in situ*, the arch and thoracic aorta were dissected free and cleaned of adherent fat and fascia. Segments were then rinsed in PBS and placed in fresh 4% paraformaldehyde overnight for paraffin embedding, or placed into 1% glutaraldehyde for subsequent electron microscopy.

Tissue processing and immunostaining

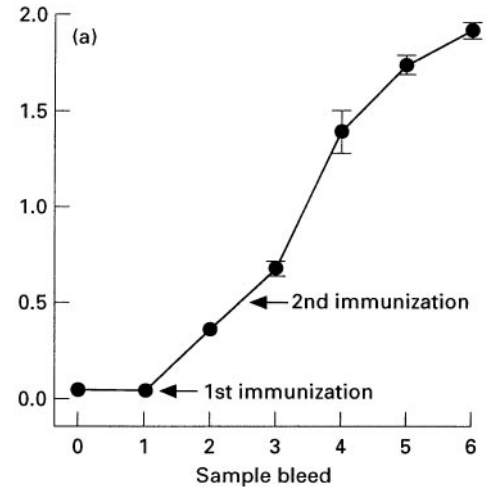
Five-micron sections of thoracic aorta at the level of the first intercostal arteries were taken for haematoxylin and eosin (H&E) staining. Adjacent sections were cut for immunostaining using monoclonal antibodies directed against rabbit alveolar macrophages (RAM-11) and smooth muscle cell actin (HHF-35), as described by Tsukada *et al*. (1986). To assess atherosclerotic involvement, the thoracic aorta was stained with Oil Red O, opened longitudinally, and photographed for subsequent morphometry.

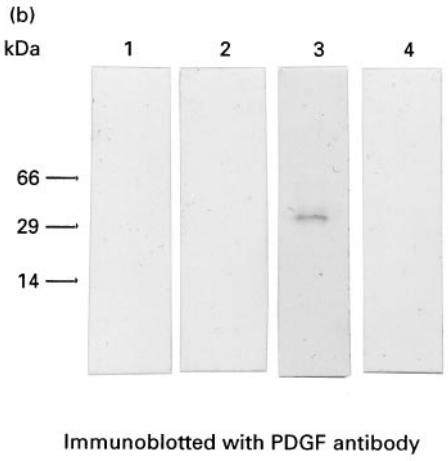
Lipid measurement

Serum cholesterol levels were measured by the CHOD-POD enzymatic-colorimetric method using a Cholesterol C-system kit (Boehringer Corp., Lewes, UK) and a Vitalab 100 autoanalyser (Vital Scientific Ltd, UK) with Precipath U and Precinorm U (Boehringer Corp., Lewes, UK) quality control material.

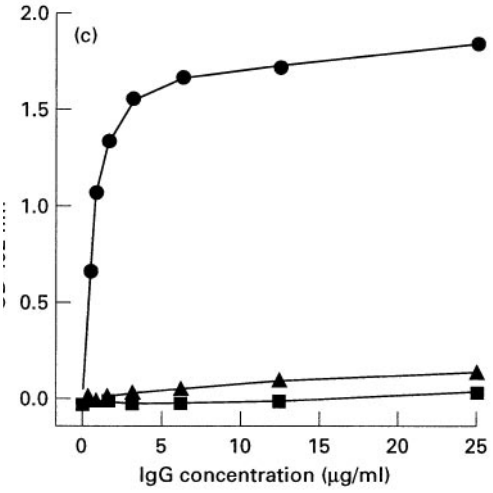
Image analysis/microscopy

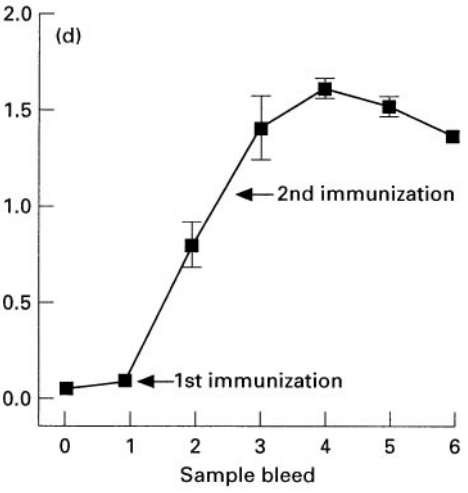
In addition to measuring the area of affected aorta, aortic atherosclerosis was assessed by measuring the intima : media ratio at the level of the first intercostal artery, and

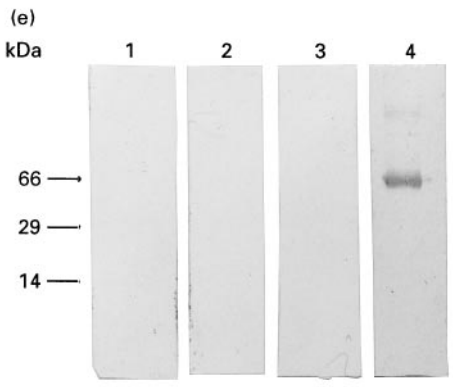




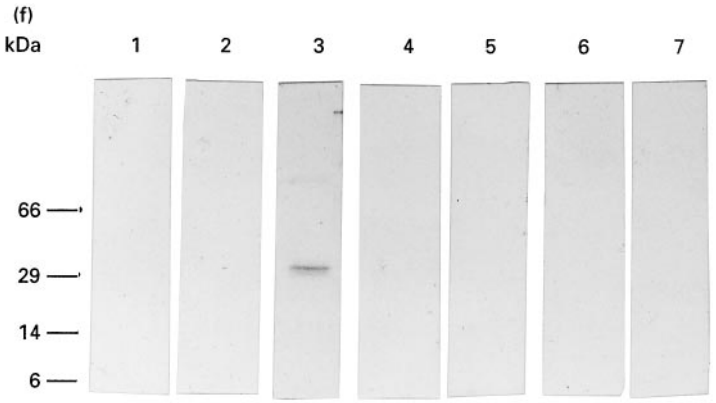
Immunoblotted with PDGF antibody







Immunoblotted with HSA antibody



Immunoblotted with PLT antibody

the percentage of the internal elastic lamina (IEL) above which lesions could be identified, using a Zeiss Axioskop microscope (Carl Zeiss Inc., Germany) equipped with a X10 Achroplan objective, a digitizing pad and an IBM 55SX PC containing a VIDS V interface card and VIDS V software (Ai Cambridge Ltd, Pampisford, UK). Measurements were performed in duplicate on three H&E stained sections. For each section the intimal and medial thickness was measured at eight separate points, the intima : media ratio calculated for each point, and the overall intima : media ratio for each section expressed as the mean of these values. The percentage of IEL covered by lesions was determined by measuring the total length of IEL and the individual lengths of atherosclerotic lesion lying above it.

Electron microscopy

Tissue segments for scanning (SEM) and transmission electron microscopy (TEM) were post fixed in 1% osmium tetroxide as described by Ferns *et al*. (1990). After dehydration in a graded ethanol series, the segments for TEM were placed in propylene oxide before embedding in Taab premix resin (Taab Laboratories Equipment Ltd, Berks., UK). Sections were cut for staining with methylene blue and areas of interest selected for thin sectioning (80 nm) using an LKB Ultramicrotome III (LKB-Bromma, Sweden). The sections were placed onto copper grids and counterstained with lead citrate and uranyl acetate and examined using a Philips 201 TEM (Philips, Eindhoven, Holland). Segments for SEM were critical point dried with CO_2 in a Biorad E3000 Critical Point Drier (VG Microtech, Uckfield, East Sussex, UK) and sputter coated with gold, using an Emscope Sputter Coater (Emscope Ltd, UK). They were then examined using a Stereoscan 180 (Cambridge Instruments, Cambridge, UK) SEM at 15 kV.

Statistical analysis

The integrated cholesterol level for each animal was calculated as the area under the serum cholesterol versus time curve. Aortic wall characteristics were compared using a one-way ANOVA with a Bonferroni correction for multiple comparisons, with a P-value <0.05 being considered to be statistically significant. Correlations between continuous variables were calculated by linear regression analysis. All statistical tests were performed using INSTAT software.

Results

Immunization with PDGF-BB and HSA, elicited endogenous specific antibodies of high titre in most animals

Immunization with recombinant PDGF-BB resulted in a rapid increase in specific antibody titres (Figure 1a) in the majority of the rabbits. Two of the seven animals immunized with PDGF-BB did not respond well even after the second booster immunization. Antibody titres in these animals, measured as optical density in the ELISA, were more than two standard deviations below the mean for the remaining group. Assessment of the antibodies by Western blotting (Figure 1b) and ELISA analysis (Figure 1c), suggested that the antibodies elicited to PDGF-BB were isoform specific. The antibodies raised

Figure 1. a, Rising plasma antibody titres to PDGF-BB in a rabbit immunized with recombinant human PDGF-BB, as measured by ELISA. Micro-titre plates (96 well, Nunc) were coated with 10 ng PDGF-BB or bFGF per well for 18 hours at 4°C under humidified conditions. The plates were rinsed 3 times in wash buffer (PBS containing 0.05% Tween-20). Non-specific protein binding was reduced by blocking with 5% milk/1% bovine serum albumin (BSA)/0.1% Tween-20 for 1 hour at 37°C after which plates were rinsed. Plasma taken from each rabbit was diluted 1 : 10 000 with PTB (BSA/Tween-20 in PBS). Aliquots (100 μl) of each were incubated in wells for one hour at 37°C. After washing, the plate was incubated with 100 μl/well 1:1000 biotinylated anti-rabbit IgG (Sigma Chemical Co.) for 60 minutes at 37°C. Unbound secondary antibody was removed by rinsing in wash buffer. Plates were then incubated with avidin/biotinylated horse-radish peroxidase (Vector Laboratories, Peterborough, UK) for 30 minutes at 30°C. Substrate (*o*-phenylenediamine, Sigma Chemical Co.) was dissolved in 0.05 M citrate/0.1 M disodium phosphate added to the wells and incubated for 5 minutes at room temperature. The reaction was terminated by adding 50 μl 3 M HCl. Absorbances were read at 492 nm on an Anthos HTII microplate reader. Data are expressed as mean ± SEM. b, Western blots of authentic PDGF-AA (Lane 1), AB (Lane 2) and BB (Lane 3) and human serum albumin (Lane 4) staining using rabbit antibodies to PDGF-BB. Blots were scanned using a Bio-Rad imaging densitometer (Model GS700) linked to a PC with Bio-Rad Molecular Analyst software. c, Cross-reactivity of rabbit anti-PDGF-BB antibody with ■, PDGF-AA; ▲, PDGF-AB and ●, PDGF-BB using an ELISA assay. Data are expressed as mean ± SEM. d, Rising plasma antibody titres to HSA in rabbit immunized against HSA measured by ELISA. Data are expressed as mean ± SEM. e, Cross-reactivity of rabbit anti-HSA antibody with PDGF-AA (Lane 1), AB (Lane 2) and BB (Lane 3), and recognition of HSA (Lane 4) using Western-blotting analysis. f, Cross-reactivity of antibody raised against rabbit platelet cytosolic protein with PDGF-AA (Lane 1), AB (Lane 2), BB (Lane 3), HSA (Lane 4), IGF-I (Lane 5), EGF (Lane 6) and TGF-β (Lane 7).

Table 1. Mean body weights, plasma cholesterol levels and aortic wall characteristics of cholesterol-fed rabbits

Group	NI	HSA	PDGF	Platelet
n	5	4	5	7
Body weight (kg)	3.77 ± 0.21	3.99 ± 0.17	3.87 ± 0.17	3.91 ± 0.12
Plasma cholesterol				
at sacrifice (mmol/l)	47.6 ± 7.9	36.9 ± 1.2	37.2 ± 7.9	40.8 ± 6.7
integrated (mmol/weeks)	433 ± 89	376 ± 21	375 ± 88	393 ± 86
Blood platelet/red cell ratio	0.37 ± 0.07	0.45 ± 0.07	0.39 ± 0.07	0.45 ± 0.04
Aortic wall characteristics				
Intimal thickness at T1 (mm)	0.192 ± 0.035	0.190 ± 0.043	0.084 ± 0.026*	0.060 ± 0.014**
Medial thickness at T1 (mm)	0.256 ± 0.034	0.180 ± 0.018	0.160 ± 0.005*	0.214 ± 0.015
I : M ratio	0.760 ± 0.111	1.075 ± 0.256*	0.280 ± 0.114**	0.257 ± 0.057***
%IEL affected at Tl	37.7 ± 5.7	58.5 ± 14.3*	21.6 ± 9.7*	12.9 ± 5.48*
% lesional aortic area	42.0 ± 1.9	64.9 ± 7.0**	26.8 ± 3.3*	24.4 ± 3.0**

Values are expressed as mean ± SEM.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to non-immunized animals using a Bonferroni test with a selected pairs correction.

against HSA were also present at high titre and did not cross-react with PDGF (Figure 1d and e). The antibodies raised against platelet cytosolic protein were found to cross-react with the BB isoform of PDGF when used for Western blotting, but did not cross-react with the other isoforms of PDGF. Nor did they cross-react with IGF-I, EGF or TGF-β (Figure 1f). Immunization of the rabbits was not associated with any significant change in circulating blood platelet counts (Table 1).

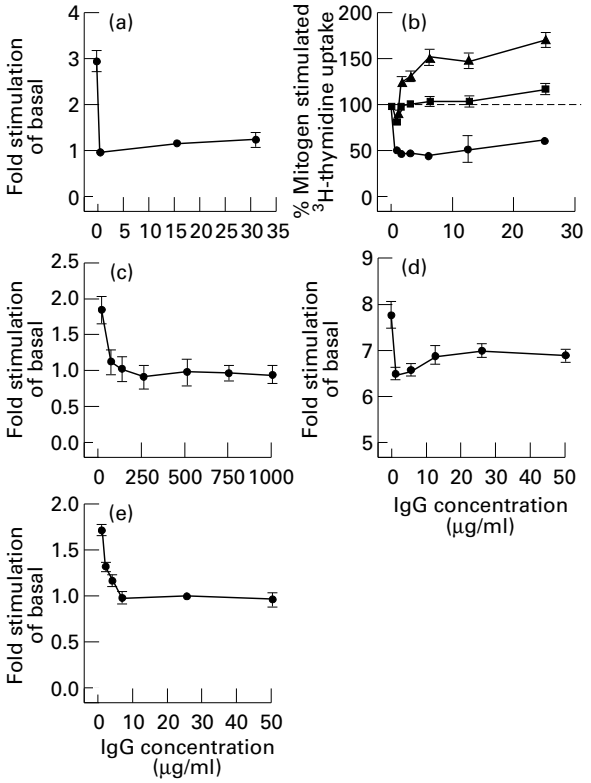


Figure 2. Effects of increasing concentrations of partially purified rabbit antibodies against PDGF-BB, on PDGF (a–c), or platelet cytosolic protein (d and e) -induced smooth muscle cell (a and d) proliferation and chemotaxis (c and e). b, The effects of a rabbit anti-PDGF-BB antibody on the mitogenic response to the three PDGF isoforms; ■, PDGF-AA; ▲, PGDF-AB; ●, PGDF-BB in 3T3-fibroblast. Data are expressed as mean ± SEM.

Endogenous antibodies to PDGF-BB and platelet cytosolic protein were neutralizing in vitro

The partially purified antibodies against PDGF-BB specifically neutralized the mitogenic effects of PDGF-BB *in vitro* at low concentrations (Figure 2a). The antibodies did not inhibit the mitogenic effects of PDGF-AA or PDGF-AB, and indeed appeared to potentiate the mitogenic effects of these two isoforms, particularly of PDGF-AB, *in vitro* (Figure 2b). This effect was probably due to the presence of small quantities of contaminating mitogenic factors in the partially purified immunoglobulin preparations. The presence of these contaminating mitogens in the partially purified antisera may also explain why they did not completely neutralize the mitogenic effects of the specific antigens. Used at high concentrations, the immunoglobulin preparations were themselves mitogenic. The antibodies against PDGF-BB were also found to inhibit the chemotactic property of PDGF-BB (Figure 2c) and rabbit platelet lysate (Figure 2e) in the micro-Boyden chamber assay, and to partially inhibit the mitogenic effect of rabbit platelet lysate (Figure 2d). The antibodies to platelet cytosolic protein similarly inhibited platelet lysate induced smooth muscle cell chemotaxis and partially inhibited the mitogenic effects of the rabbit platelet lysate (Figure 3a and b).

Cholesterol feeding caused a significant increase in plasma cholesterol

The 2% cholesterol diet was well tolerated by most

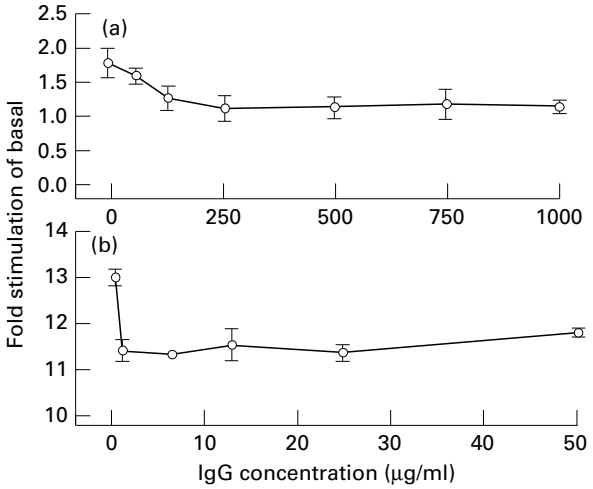


Figure 3. Effects of increasing concentrations of partially purified rabbit antibodies against platelet cytosolic protein on a, platelet lysate induced smooth muscle cell chemotaxis and b, proliferation. Data are expressed as mean ± SEM.

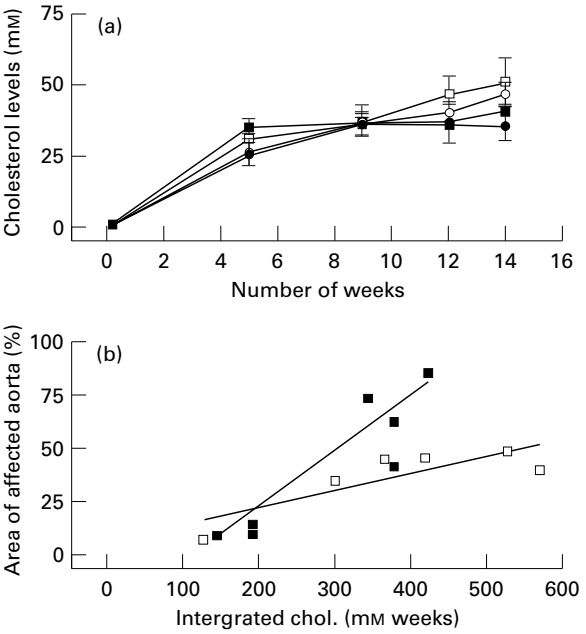


Figure 4. Serum cholesterol levels over time a, in the four groups of rabbits ●, PDGF ($n = 5$); ○, PLT($n = 7$); ■, HSA ($n = 4$); □, NI ($n = 5$) fed a 2% cholesterol enriched diet; and b, the relationship between aortic lesion development and integrated serum cholesterol levels in □, non-immune animals ($n = 6$) and ■, rabbits immunized with HSA ($n = 7$). Data are expressed as mean ± SEM. Linear regressions were calculated using Instat software: HSA, $r^2 = 0.830$, $P = 0.004$; NI, $r^2 = 0.686$, $P = 0.042$.

animals for the duration of the experiment, and caused a significant increase in plasma cholesterol levels within 5 weeks of commencing the diet (Figure 4a). In many of the animals plasma cholesterol levels continued to rise further over the subsequent weeks. However, in five of the animals (three immunized against HSA, one immunized against platelet cytosolic protein, and one non-immune), either the plasma cholesterol response to the high cholesterol diet was poor, or else they could not tolerate the diet for the whole duration of the experiment. Although the data from these animals could not be used for the group comparisons, they were used to assess the effects of integrated cholesterol levels on the extent of lesion development (Figure 4b).

Cholesterol feeding caused the development of atherosclerotic lesions in the aorta

Fatty streaks developed in all groups of animals (Figure 5). Lesions were most severe in the aortic arch, and were less evident in the distal thoracic aorta, where lesions were related to intercostal artery branch points (Figure 5). Electron microscopy and immunocytochemistry (Figure 6b–d) confirmed the accumulation of numerous macrophage-derived foam-cells beneath an intact endothelium. Migrating medial smooth muscle cells were also identified by electron microscopy (data not shown).

Aortic lesion involvement was related to integrated plasma cholesterol levels

There was a direct relationship between the area of aorta staining positive with Oil Red-O and integrated plasma cholesterol levels in the non-immune animals and those immunized with HSA (Figure 4b). Low integrated cholesterol levels would therefore confound our analysis of lesion size versus antibody titre. For this reason the analysis of the effects of endogenous antibodies on lesion development was confined to groups of animals that were reasonably well matched for integrated plasma cholesterol levels (Figure 7a).

Endogenously elicited antibodies to PDGF-BB and platelet cytosol inhibited aortic lesion development

Immunization with HSA was associated with a significant increase in the percentage of aorta staining positive with Oil Red-O, whereas immunization with either PDGF-BB or platelet cytosolic protein was associated with a significant inhibition in the percentage of aorta affected compared to non-immune animals (Figure 7b). A similar pattern of response was observed when the extent of lesion development was quantitated as the ratio of intimal : medial thickness at the level of the first

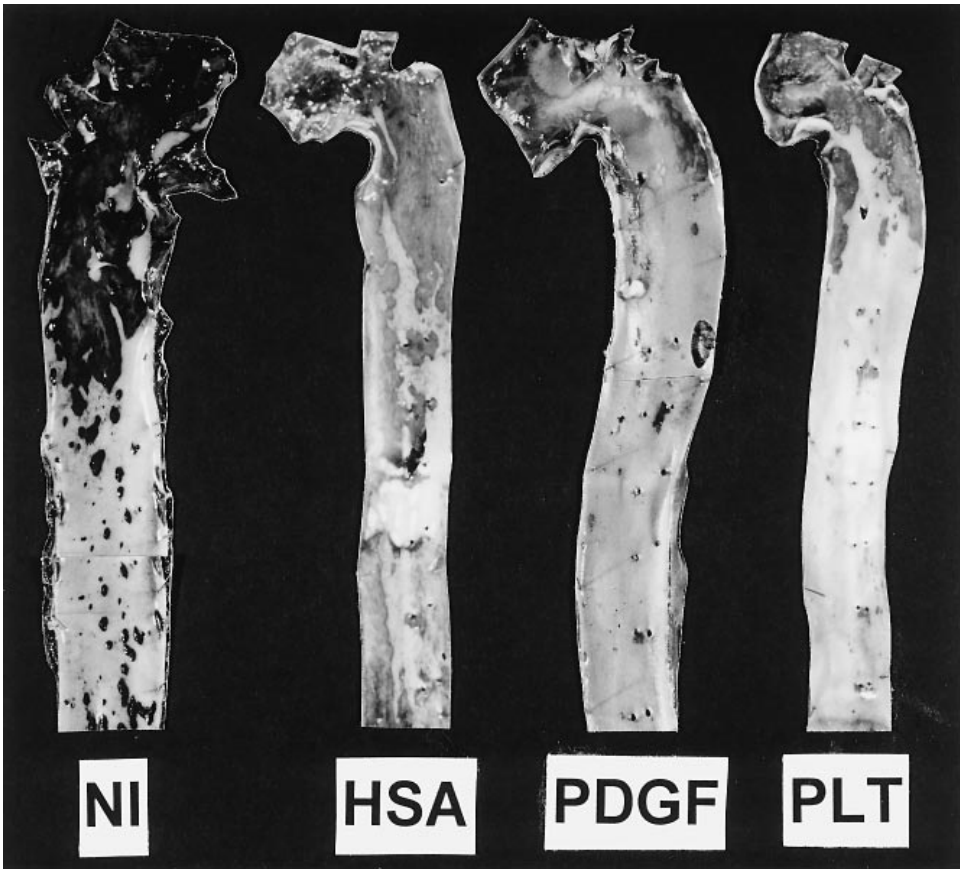


Figure 5. En-face views of representative aortae from rabbits fed a 2% cholesterol enriched diet for approximately 3 months in animals that were immunized against human serum albumin (HSA), PDGF-BB, or platelet cytosolic protein (PLT), or injected with Freund's adjuvant alone (NI).

intercostal branch point (Figure 7c), or as the percentage of intima above the internal elastic lamina affected by lesions (Figure 7d and Table 1). In the animals immunized against PDGF-BB, there was an inverse relation between the extent of aortic lesion development and specific antibody titres ($P < 0.05$) (Figure 8a), whereas in the animals immunized with HSA there was a positive association (Figure 8b).

Discussion

The role of PDGF in atherogenesis

PDGF is one of the principal platelet associated factors responsible for mitogenic effects on mesenchymal cells, including vascular smooth muscle cells (Ross *et al.* 1974). More recent work has shown that PDGF is dimeric, consisting of homo or hetero-dimeric combinations of two polypeptide chains (Johnsson *et al.* 1984). Analysis of sera from different species has shown that the relative importance of PDGF as a serum mitogen varies between species, and that the amounts of each of the three PDGF isoforms also differ between species (Bowen-Pope *et al.* 1989). Platelets are not the only source of PDGF. It has now been established that various isoforms of PDGF may be expressed by endothelial cells, smooth muscle cells and macrophages (reviewed by Ross 1993). Vascular smooth muscle cells also express receptors for PDGF, and may hence respond in an autocrine, or paracrine fashion to this growth factor (Bowen-Pope *et al.* 1985). In addition to its mitogenic properties, PDGF is a potent chemoattractant. The precise role of chemotaxis in atherogenesis is uncertain; however, the rate of cell proliferation is low in human atherosclerotic lesions (Gordon *et al.* 1990; Rekhter & Gordon 1994), whereas in models of accelerated atherosclerosis, chemotaxis appears to be an important process (Ferns *et al.* 1991; Rutherford *et al.* 1996; Jawien *et al.* 1992). The neo-intimal smooth muscle cells may contribute to the recruitment and retention of

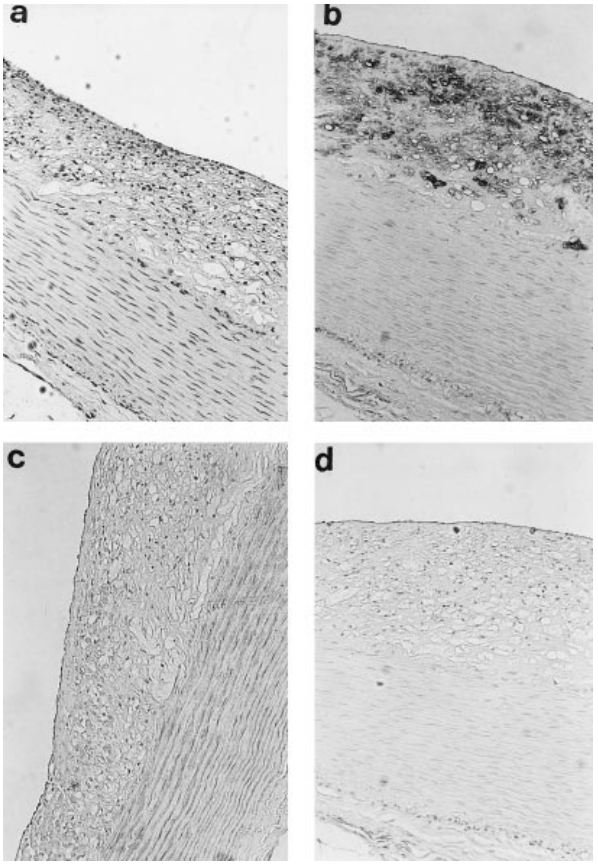


Figure 6. a, Haematoxylin and eosin (H&E) stained section of aorta at the level of the first intercostal artery branch point from a rabbit fed on a 2% cholesterol diet for 3 months. ×200. Serial sections stained with antibodies against b, macrophages (RAM-11); c, smooth muscle cells (HHF-35), or d, a control section stained in the absence of primary antibody.

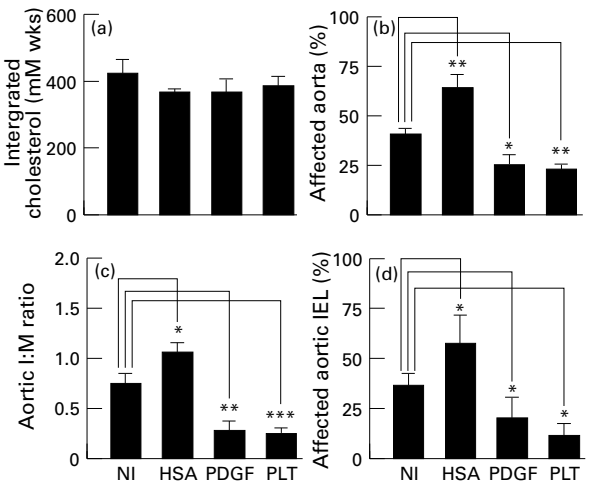


Figure 7. a, Integrated serum cholesterol levels in the four groups of experimental rabbits. The effects of endogenous antibody production on aortic lesion development, assessed as b, percentage Oil Red-O positive of total aortic area (ascending, arch and thoracic aorta); c, aortic intima : media ratio at the level of the first intercostal artery branch point; and d, percentage affected aorta above the internal elastic lamina at the same level. Values are expressed as ±s.e.m. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared to non-immunized animals using a Bonferroni test with a selected pairs correction.

circulating monocytes through their release of chemotactic factors such a MCP-1 (Takeya *et al.* 1993) and expression of cell adhesion molecules (Poston *et al.* 1992). Extracellular matrix production also contributes to intimal lesion size, and Papakonstantinou *et al.* (1995) have recently reported that PDGF stimulates the secretion of hyaluronan, a major constituent of the extracellular matrix, and Suzuki *et al.* (1995) have shown this is mediated via protein kinase C. In the presence of hypercholesterolaemia, uptake of modified LDL via a scavenger receptor mediated pathway may also play an important role in atherosclerotic lesion development. Gong and Pitas (1995), Inaba *et al.* (1992) and Fuhrman *et al.* (1992) have reported that platelet proteins, including PDGF-BB, enhance scavenger receptor activity in vascular smooth muscle cells and macrophages. These data suggest that PDGF may be a major player in early atherogenesis. Our data support this hypothesis, whilst indicating that of PDGF's three isoforms, PDGF-BB may be of particular importance in the cholesterol-fed rabbit. All three indices of aortic atherosclerosis were significantly inhibited in rabbits immunized against PDGF-BB.

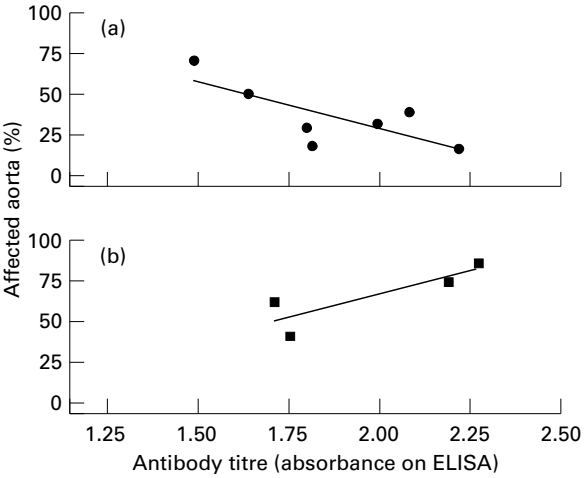


Figure 8. Relation between plasma antibody titres against a, PDGF-BB, $r^2 = 0.568$, $P = 0.05$; b, HSA, $r^2 = 0.727$, $P = 0.147$ and Oil Red-O staining lesions in cholesterol-fed rabbits.

The possible involvement of other platelet associated factors in atherogenesis

In addition to PDGF, platelets contain several other potentially mitogenic growth factors, including insulin-like growth factor-I, epidermal growth factor and transforming growth factor beta. In the Western blot analysis of the anti-platelet antibody we found it did cross-react with recombinant PDGF-BB, but not with the other isoforms of PDGF, or with IGF-I, TGF-β or EGF. Immunization of rabbits against platelet cytosolic protein was associated with significant reduction in atherosclerotic lesion formation, similar in magnitude to that seen in the animals immunized against PDGF-BB alone. The anti-platelet antibody was found to cross-react with PDGF-BB, and this may partly explain its efficacy in reducing atherosclerotic lesion formation, although its effects on other platelet associated factors cannot be excluded. These data again support the notion that PDGF-BB is indeed an important mediator of the atherogenic process.

Effects of immunomodulation of atherogenesis

T-lymphocytes have been shown to be present in atherosclerotic lesions of cholesterol-fed non-human primates (Masuda & Ross 1990), rabbits (Roselaar *et al*. 1995) and in human lesions (Stemme *et al*. 1995). Their precise role is unclear, but it is possible they are involved in a local immunological response mounted against modified lipoprotein constituents. A proportion of these lymphocytes exhibit activation markers, and are probably capable of secreting factors, such as gamma interferon (Hansson *et al*. 1989). These factors may modify the activity of cells within the plaque, and enhance the atherogenic process. Previous work has indicated that stimulation of the immune system results in enhanced atherosclerotic lesion formation in rabbits fed a cholesterol, or lipid-rich diet (Minick *et al*. 1996; Minick & Murphy 1973). Our data are consistent with these previous studies. Immunization with HSA caused a significant increase in atherosclerotic lesion formation in the aortae of cholesterol-fed rabbits. Xu *et al*. (1992) have previously reported that, in the normocholesterolaemic and cholesterol-fed rabbit, immunization with heat shock protein enhanced atherosclerotic lesion formation, suggesting that heat shock protein may modulate the atherosclerotic process. The authors also reported that immunization with ovalbumin or atherosclerotic lesion protein did not significantly alter the extent of aortic lesion in the normocholesterolaemic rabbit. Alving *et al*. (1995) have recently found that rabbits immunized against crystalline cholesterol developed antibodies that recognized the plasma lipoproteins, very low density lipoprotein (VLDL) and intermediate density lipoprotein (IDL), and their presence appeared to lower cholesterol levels in cholesterol-fed animals. Lowering of plasma cholesterol by this means was associated with reduced aortic atherosclerosis.

Possible clinical implications

Our data support previous reports regarding the importance of PDGF-BB in the evolution of atherosclerotic plaque formation. PDGF-B chain may be derived from several sources, including platelets, macrophage-derived foam-cells and dysfunctional endothelium. Extrapolations from experimental animal models, such as the cholesterol-fed rabbit, to man, should be made with caution because there do appear to be interspecies differences in the cellular distribution and expression of the three PDGF isoforms. For this reason, it would appear worthwhile to extend our model of endogenous antibody production to other forms of experimentally induced atherosclerosis.

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