Tyrosine phosphorylation of platelet derived growth factor β receptors in coronary artery lesions: implications for vascular remodelling after directional coronary atherectomy and unstable angina pectoris

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Abstract

Background—Growth factors such as platelet derived growth factor (PDGF) have been postulated to be important mediators of neointimal proliferation observed in atherosclerotic plaques and restenotic lesions following coronary interventions. Binding of PDGF to its receptor results in intrinsic receptor tyrosine kinase activation and subsequent cellular migration, proliferation, and vascular contraction.

Aims—To investigate whether the concentration of PDGF β receptor tyrosine phosphorylation obtained from directional coronary atherectomy (DCA) samples correlate with atherosclerotic plaque burden, the ability of diseased vessels to remodel, coronary risk factors, and clinical events.

Methods—DCA samples from 59 patients and 15 non-atherosclerotic left internal thoracic arteries (LITA) were analysed for PDGF β receptor tyrosine phosphorylation content by receptor immunoprecipitation and antiphosphotyrosine western blot. The amount of PDGF β receptor phosphorylation was analysed in relation to angiographic follow up data and clinical variables.

Results—PDGF β receptor tyrosine phosphorylation in the 59 DCA samples was greater than in the 15 non-atherosclerotic LITA (mean (SD) 0.84 (0.67) v 0.17 (0.08) over a control standard, p < 0.0001). As evaluated by stepwise regression analysis, incorporation of both PDGF β receptor tyrosine phosphorylation and immediate gain correlated strongly (adjusted r² = 0.579) with late loss, although PDGF β receptor tyramine phosphorylation alone correlated poorly with late loss. Multivariate regression analysis of coronary risk factors and clinical events revealed unstable angina as the most significant correlate of PDGF β receptor tyrosine phosphorylation (F value 20.009, p < 0.0001).

Conclusions—PDGF β receptor tyrosine phosphorylation in atherosclerotic lesions is increased compared with non-atherosclerotic arterial tissues. The association of PDGF β receptor tyrosine phosphorylation with immediate gain strongly correlates with vascular remodelling. PDGF β receptor tyrosine phosphorylation correlates with unstable angina pectoris.

Keywords: PDGF receptors; atherosclerosis; directional coronary atherectomy; restenosis

The development of coronary artery restenosis continues to be a major limitation of percutaneous transluminal coronary angioplasty (PTCA). Clinical and basic research have implicated several important factors in the pathogenesis of restenosis including: smooth muscle proliferation, thrombus formation, overproduction of extracellular matrix, progression of the underlying atherosclerotic lesion, and failure of vessel remodelling. Of note, intimal hyperplasia due to smooth muscle proliferation appears to be a common and important event in the process of restenosis.

Several growth factors have been shown to stimulate vascular smooth muscle cell proliferation and may be involved in restenosis: platelet derived growth factor (PDGF), fibroblast growth factor, transforming growth factor β, angiotensin II, and insulin-like growth factor 1. Because of its potent activity as a mitogen and chemoattractant as well as its presence in neointimal tissues and atherosclerotic plaques, PDGF has been postulated to be an important factor responsible for vascular smooth muscle proliferation. In addition, PDGF stimulates vascular contraction, and may contribute to the enhanced vasoreactivity of certain atherosclerotic vessels.

Several groups have studied the expression of the PDGF receptor and its ligand within human atherosclerotic tissue. Using in situ hybridisation, PDGF A and B chain mRNAs were found in mesenchymal appearing intimal cells and endothelial cells, respectively. However, Barrett et al. reported that PDGF A and B chains were also highly expressed in non-atherosclerotic artery, suggesting that PDGF A and B chain mRNA expression did not necessarily cause smooth muscle cell proliferation in atherosclerosis. In contrast, both α and β receptors of PDGF are localised predominantly in the atherosclerotic plaque intima, while in vessels of normal tissues PDGF β receptors were not expressed or only
minimally expressed. However, the activity of PDGF in vivo in atherosclerotic plaques is unknown. PDGF exerts its biological effects through high affinity interactions with two subtypes (α and β) of PDGF receptors. PDGF A chain interacts only with PDGF α receptor, while PDGF A chain interacts with α and β receptors. PDGF B chain is more potent than PDGF B chain as a mitogen and chemoattractant for vascular smooth muscle cells via its interaction with the PDGF β receptor. The activation of the intrinsic tyrosine kinase activity of the PDGF receptor by PDGF ligand results in receptor autophosphorylation at critical tyrosine residues and activation of second messenger pathways necessary for the induction of gene expression, DNA synthesis, mitogenesis, and vascular contraction. Therefore, to investigate the role of PDGF in atherosclerosis and vascular stenosis after percutaneous transluminal coronary angioplasty (PTCA), we hypothesised that the measurement of the extent of PDGF β receptor tyrosine phosphorylation in cells and tissues should provide important information about the PDGF β receptor and its activation in vivo. By evaluating tyrosine phosphorylation of PDGF receptors, we recently observed that in a rat carotid balloon injury model, the activation of PDGF receptors correlated with neointima formation.

The use of directional coronary atherectomy (DCA) has allowed investigators to sample tissue from human intravascular plaques. We measured the extent of tyrosine phosphorylation of PDGF β receptor in atherectomy samples, and analysed the relation between the extent of tyrosine phosphorylation of PDGF β receptor in atherectomy specimens and the degree of subsequent vascular remodelling, as well as clinical variables.

Methods

PATIENTS AND AHERETOMY PROCEDURES

Coronary atherectomy specimens were obtained with the Simpson AtheroCath (Devices for Vascular Interventions, Redwood, California, USA) from 59 patients anatomically and clinically suitable for the procedure between 10 October 1993 and 6 September 1994 at Mitsui Memorial Hospital. Patients who showed enzymatic or electrocardiographic evidence of acute myocardial infarction were excluded. Premedication with aspirin (325 mg/day) was started 24 hours before the procedure, and a 10 000 U bolus of heparin was given after insertion of the 10 F arterial sheath and supplemented as needed to maintain the activated clotting time at > 250 seconds. Approximately 20 mg of tissue were removed in each procedure; one half of which was used for determination of tyrosine phosphorylation of PDGF β receptor, and the other for light microscopy. Before DCA was performed, we explained to the patients the objectives, procedures, protocols, and possible complications involved. Informed written consent was obtained from all patients who underwent DCA. The study protocol had been approved by our institutional review board. As a normal control, a segment of internal thoracic artery was obtained from 15 patients who underwent coronary bypass graft operation. None of the internal thoracic arteries had significant intimal thickening by microscopic examination.

Determination of tyrosine phosphorylation of PDGF receptor

Each atherectomy specimen was removed promptly from the atherectomy collection chamber and immediately stored at −80°C. Surgically resected internal thoracic arteries were transported at 4°C to the laboratory and immediately processed for immunoprecipitation. Specimens were homogenised at 4°C in 300 ml of a buffer containing 50 mM Tris-HCl pH 8.0, 120 mmol/l NaCl, 0.5% Nonidet-P 40, 100 mmol/l NaF, 1 mmol/l NaVO₃, 0.1% sodium dodecylsulphate (SDS), 2 mmol/l EGTA, 80 mg/ml each of leupeptin and aprotinin, and 0.6 mmol/l phenylmethylsulphonyl fluoride. The homogenate was cleared by centrifugation at 10 000 xg for five minutes at 4°C. After an aliquot of the supernatant was used for the determination of protein content, 250 µg of cell protein per sample was subjected to immunoprecipitation using antihuman PDGF β receptor antibody (UBI, Lake Placid, New York, USA). The immune complex was collected by incubation with protein A sepharose (Pharmacia). The immunoprecipitates were subjected to 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under a reducing condition. The separated proteins were electrotransferred to an Immobilon-P membrane (Millipore) at 160 mA for 30 minutes. Non-specific binding of antibodies was blocked by incubating a membrane with 3% (wt/vol) bovine serum albumin in Tris buffered saline (137 mmol/l NaCl, 20 mmol/l Tris-HCl, pH 7.6). The membrane was probed with anti-phosphotyrosine antibody (clone 4G10) (UBI), and visualised by the ECL chemiluminescence method as described previously. The extent of PDGF β receptor phosphorylation was measured by a scanning densitometer (Quantity One; PDI Inc, New York, USA). The background could be subtracted easily and objectively using the rolling method software analysis provided with Quantity One. The PDGF β receptor phosphorylation value of

<table>
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<th>Sample</th>
<th>Wet weight (mg)</th>
<th>Protein content (µg)</th>
<th>Raw value (OD)</th>
<th>Value per 10 mg tissue</th>
<th>Value per 100 µg protein</th>
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</tr>
<tr>
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<td>23.8</td>
<td>286</td>
<td>0.033</td>
<td>0.0138</td>
<td>0.0115</td>
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Three segments were cut from a gastroepiploic artery, weighed, homogenised, assayed for protein content, and analysed for tyrosine phosphorylation of PDGF β receptor. OD, optical density.
atherectomy specimens and left internal thoracic arteries was expressed as the ratio of densitometric value (OD) of the specimen to the control standard value (OD) of PDGF receptor tyrosine phosphorylation of PDGF B stimulated human fibroblasts.

ANGIOGRAPHIC ANALYSIS

Follow up angiography was performed on symptom free patients three to six months after DCA. However, patients with chest pain or positive exercise test underwent angiography earlier. Angiographic analysis was done with a computer assisted electronic caliper system (Cathex Coronary Artery Measurement System; Cathex Inc, Tokyo, Japan). Measurements were made in a single projection using a frame that showed the clearest and severest stenosis. Whenever possible, all three measurements (before DCA, immediately after DCA, and at follow up) were performed with the same camera angulation to allow a more precise comparison. The amount of immediate gain was defined as the difference in luminal diameter at the lesion before and immediately after atherectomy. The amount of late loss was defined as the absolute change in luminal diameter immediately after atherectomy and at follow up. Restenosis was defined as stenosis of more than 50% at the DCA site at the time of follow up angiography.

STATISTICAL ANALYSIS

Data are reported as mean (SD). Statistical analysis was performed with the StatView 4.0 package (ABACUS Concepts, Berkeley, California, USA). Differences were analysed with unpaired two-tailed Student’s t test, Mann-Whitney test, or by linear regression analysis, as appropriate. Independent determinants of the linear outcomes of interest were constructed by simple and multivariate regression analyses. The final model that contained the independent determinants in each analysis was described as a linear combination of intercept and β terms. A p value of < 0.05 was considered statistically significant.

The relation between the extent of tyrosine phosphorylation of PDGF β receptor and clinical variables were analysed by simple and stepwise regression analysis (a forward selection procedure) as described previously. The variables analysed were risk factors for coronary artery disease—sex, hypertension (blood pressure that required treatment before DCA), diabetes mellitus, smoking, family history of coronary artery disease, obesity (> 20% ideal body weight), restenotic lesions, unstable angina, raised serum cholesterol concentration, and age at the time of DCA. Any variable with a partial F value of ≥ 4.0 was included in the regression analysis, and any previously entered variables with a partial F value of < 4.0 were removed from the analysis.

Results

BASELINE CLINICAL CHARACTERISTICS OF THE STUDY PATIENTS

The study population comprised 59 patients (44 men and 15 women) with a mean age of 62 years. Twenty patients presented with clinically unstable angina. However, their condition stabilised with medical treatment by the time they were subjected to the study atherectomy. The remaining 39 patients had stable angina. A single lesion from each patient was used for the study. Of the 59 atherectomy specimens obtained, 48 were from the left anterior descending coronary artery, seven from the right coronary artery, two from protected left main trunks, and one from a saphenous vein graft. Thirty of the specimens were from primary lesions to which a percutaneous coronary intervention was attempted for the first time. The other 29 specimens were from restenotic lesions. The average interval between the prior intervention and the study atherectomy in the restenosis lesion group was 4.5 months (range 2–8). Seven of 29 restenotic lesions developed after previous DCA, the other 22 after conventional balloon angioplasty.

ANGIOGRAPHIC OBSERVATIONS

The mean (SD) angiographic severity (percentage reduction in diameter) of target lesions was 66(13)%, and the mean reference diameter was 2.85 (0.58) mm. Both device success and lesion success were obtained in all patients, resulting in a residual stenosis of −3(21)%. The mean immediate gain of lumen diameter in these patients was 2.00 (0.74) mm (range 0.75–3.56). Angiographic follow up data were obtained for 49 patients (83%) a mean of 121 days after the study atherectomy (range 79–185). Fifteen of the 49 vessels showed restenosis (stenosis of more than 50%) at the site of atherectomy, with the average ratio of late loss...
to immediate gain of lumen diameter (the loss index) of 0.93 (0.23). The 34 patients without restenosis had an average loss index of 0.34 (0.20). All 15 patients with restenosis underwent an additional percutaneous coronary intervention; the remaining 34 patients without restenosis were treated with drugs.

### ANALYSIS OF TYROSINE PHOSPHORYLATION OF PDGF β RECEPTOR IN DCA SPECIMENS

Attempts to evaluate PDGF β receptor protein quantitatively in DCA specimens were inconclusive due to the limited quantity of protein present in DCA specimens. Therefore, we focused on the tyrosine phosphorylation of PDGF β receptor, which more accurately reflects PDGF β receptor “activity”.

To evaluate whether determination of PDGF β receptor tyrosine phosphorylation in arterial tissues can be performed quantitatively, we measured tyrosine phosphorylation of human non-atherosclerotic artery (gastroepiploic artery) by immunoprecipitation of PDGF β receptors from arterial homogenates and western blotting using antiphosphotyrosine antibody. Tyrosine phosphorylation of PDGF β receptor in three arterial segments of different sizes were similar when the values were expressed as an optic density value per a constant tissue weight or per a constant protein amount, indicating that phosphorylation of PDGF β receptors can be measured quantitatively (table 2).

A significant amount of tyrosine phosphorylation of PDGF β receptor was detected in all of the DCA specimens and control internal thoracic arteries. As shown in a western blot of 10 representative atherectomy specimens (fig 1), the extent of tyrosine phosphorylation of PDGF β receptor was variable (range 0.05–3.99) (fig 2). The mean value of PDGF β receptor phosphorylation for 59 samples was significantly greater in the DCA specimens than in non-atherosclerotic internal thoracic arteries (0.84 (0.67) v 0.17 (0.08), p < 0.0001). These results indicate that in stenotic lesions of coronary arteries, activated PDGF β receptors are present in varying amounts.

Of the 49 patients who underwent follow up angiography, the extent of PDGF β receptor tyrosine phosphorylation was significantly greater in the 15 individuals that developed restenosis than in the 34 subjects who did not (1.31 (0.87) v 0.63 (0.43); p < 0.02). In the 49 patients with follow up angiography, the extent of tyrosine phosphorylation of PDGF β receptor correlated with three measures of restenosis: the loss index ratio of luminal diameter (r = 0.664, p < 0.0001), the amount of late loss of lumen diameter (r = 0.315, p = 0.0269), and percentage stenosis (r = 0.499, p = 0.0002). There was no significant difference in the tyrosine phosphorylation of PDGF β receptor between specimens from 30 primary lesions and from 29 restenotic lesions. In the present study, however, immediate gain was not correlated with PDGF β receptor tyrosine phosphorylation (r = 0.201, p = 0.17), indicating that the good correlation of the loss index ratio with PDGF receptor phosphorylation is not due to the presence of immediate gain in the loss index ratio.

Previous studies revealed that the amount of late loss of luminal diameter observed on angiography is strongly influenced by the immediate gain of lumen diameter produced by coronary interventions.24 26 27 These observations may...
Of lumen diameter (adjusted $R^2 = 0.080$) in the 49 patients as evaluated by simple regression analysis (fig 3A). The amount of immediate gain of lumen diameter after DCA also correlated better with late loss of lumen diameter (adjusted $R^2 = 0.375$) than PDGF β receptor tyrosine phosphorylation (fig 3B), which confirms previous observations.²⁶⁻²⁷ The combination of the extent of PDGF β receptor phosphorylation and the amount of immediate gain in lumen diameter even strongly correlated (adjusted $R^2 = 0.579$) with the extent of late loss of lumen diameter as evaluated by multivariate regression analysis (fig 3C). Thus, patients with both a high level of PDGF β receptor tyrosine phosphorylation and a large immediate gain at the time of initial atherectomy had a greater degree of late loss after atherectomy than did patients with one or none of these factors.

**RELATION OF CLINICAL VARIABLES TO THE EXTENT OF PDGF β RECEPTOR PHOSPHORYLATION IN DCA SPECIMENS**

Linear regression analysis techniques were used to investigate which clinical variables are associated with tyrosine phosphorylation of PDGF β receptor (table 2). Simple regression analysis revealed significant correlations between the extent of PDGF β receptor phosphorylation and unstable angina (p < 0.0001), age (p = 0.0012), and tobacco use (p = 0.0391). Stepwise regression analysis revealed unstable angina and cholesterol as significant independent variables (table 2). On the basis of the standardised regression coefficients, the strongest determinant of the extent of PDGF β receptor phosphorylation among the coronary risk factors and clinical events was unstable angina. The mean value of the extent of PDGF β receptor tyrosine phosphorylation in DCA specimens from the patients with unstable angina was 1.43 (0.98), which was significantly (p < 0.002) higher than in specimens from the 39 patients with stable angina (0.62 (0.48)) (fig 4).

**Discussion**

This study is the first to document tyrosine phosphorylation of PDGF β receptor in vivo (a measure of PDGF β receptor activation) in atherectomy specimens and in normal arteries. The three major findings in the present study are: PDGF β receptor tyrosine phosphorylation in atherosclerotic lesions is increased compared with non-atherosclerotic arteries; the association of PDGF β receptor tyrosine phosphorylation with immediate gain correlates with vascular remodelling after DCA; and among a number of coronary risk factors and clinical events examined, unstable angina is significantly associated with the increased extent of PDGF β receptor tyrosine phosphorylation.

The observation of that the extent of tyrosine phosphorylation of PDGF β receptor was increased in most specimens from atherosclerotic lesions of coronary arteries compared with non-atherosclerotic internal thoracic arteries implies increased local activity of PDGF in
atherosclerotic plaques. In atherosclerotic lesions, PDGF B chain, a preferred ligand for PDGF β receptor, was reported to be produced and released from infiltrating monocytes/macrophages, endothelium, and/or adhering platelets. The local release of PDGF B chain at atherosclerotic lesions is presumably increased in patients with greater phosphorylation of PDGF β receptor in atherectomy specimens. The observed tyrosine phosphorylation of PDGF β receptor is unlikely to be caused by the DCA procedure itself, because we found no correlation between PDGF β receptor tyrosine phosphorylation and immediate gain of the lumen diameter. Locally released PDGF is very likely an important growth factor leading to intimal proliferative response in the injured vascular wall. Increased local release of PDGF in injured stenotic lesions could cause augmented proliferation. Moreover, it is reported that there are compensatory enlargement mechanisms associated with neointimal formation after coronary interventions, and this geometric remodelling is important to luminal narrowing after PTCA. One explanation for this observation is that PDGF increases the cytosolic free calcium concentration and causes a concentration-dependent contraction of the artery. Thus, it appears reasonable to conclude that greater PDGF activity prevents the enlargement mechanism of vascular remodelling and contributes to restenosis after PTCA.

It was previously demonstrated that the amount of immediate gain of lumen diameter after a coronary intervention correlates with the amount of late loss of lumen diameter. The results of the present study revealed that the extent of PDGF β receptor tyrosine phosphorylation also correlated with the amount of late loss of lumen diameter, although weaker than immediate gain of lumen diameter (fig 3A and B). It is now generally recognised that although late loss increases with immediate gain of lumen diameter, increased immediate gain still produces a net beneficial effect of reducing restenosis because the amount of late loss is only a fraction of the amount of immediate gain. However, it is still difficult to predict the probability of restenosis for individual patients based on the amount of late loss. The data in this study may suggest that the local PDGF activity reflected by the tyrosine phosphorylation level of PDGF β receptor in atherectomy specimens is one biological influence on restenosis. The observation that PDGF β receptor phosphorylation and immediate gain correlated better with angiographic late loss than either PDGF β receptor tyrosine phosphorylation or immediate gain alone (fig 3C) supports this notion.

Although we could not accurately measure the amount of PDGF β receptor protein by western blotting, we believe that PDGF β receptor tyrosine phosphorylation, as measured by phosphotyrosine content of immunoprecipitated PDGF β receptors, is an accurate predictor of total PDGF β receptor activity for several reasons: tissue PDGF β receptor number is a critical determinant of PDGF mediated activity and receptor immunoprecipitation has been determined to be quantitatively (table 1) after PDGF ligand binding to the PDGF β receptor, receptor activation occurs by tyrosine phosphorylation of the receptor. Thus, the phosphotyrosine content of the PDGF β receptor reflects tissue PDGF β receptor “activation”. Therefore, phosphotyrosine content of the PDGF β receptor is a better predictor of tissue PDGF β receptor activity as it reflects both receptor number and activation by PDGF–ligand binding. As equal quantities of protein from DCA samples were analysed for PDGF β receptor phosphotyrosine content, the phosphotyrosine levels of PDGF β receptors in these samples are an accurate correlate of vascular remodelling and clinical events. By applying a similar technique to rat carotid balloon injury model, we recently observed that PDGF β receptor tyrosine phosphorylation is increased during the growing phase of the neointima and that suppression of neointima formation is accompanied by a decrease in PDGF β receptor tyrosine phosphorylation.

It is important to emphasise that the extent of PDGF β receptor activation in atherectomy specimens from patients with unstable angina was higher than in those with stable angina (fig 4). Numerous clinical, anatomical, and procedural variables have been proposed as predictors of restenosis after successful PTCA. Among the clinical variables, diabetes mellitus and unstable angina have been mentioned most frequently. However, few studies have addressed the molecular mechanisms of the restenosis process in unstable angina. Angiography, angiography, and histological studies have demonstrated that the most important pathological feature of unstable angina is coronary thrombosis. Plaque disruption resulting in platelet aggregation, and subsequent thrombus formation are highly associated with unstable angina and acute myocardial ischaemia. Moreover, thrombi are rich in platelets and monocytes/macrophages, both of which are associated with the biological activities of PDGF in the vascular wall. In addition, recently it has been reported that increasing amounts of macrophages have been detected in the plaque shoulder regions of histological sections from patients with acute coronary syndromes. Therefore, PDGF released by platelets in coronary thrombi and macrophages in plaques, walls may activate vascular PDGF β receptor and therefore be responsible, at least in part, for the association between unstable angina and restenosis after a percutaneous transluminal coronary intervention.

This study was intentionally limited to describing an important new relation between the loss index and PDGF β receptor tyrosine phosphorylation, regardless of whether the lesions were de novo or restenotic. In interpreting our findings, several limitations need to be mentioned. First, the mechanism of restenosis may differ in patients with unstable and stable angina, and in de novo and restenotic lesions. However, Kuntz et al have reported that the
late loss in lumen diameter varied directly with immediate gain, although they include 41% prior restenosis lesions in their study population. Another limitation is that we could not accurately measure the level of PDGF β receptor protein by western blotting due to the limited amount of protein. However, as equal amounts of total protein from DCA and LITA control samples were analysed, we believe that PDGF β receptor phosphotyrosine content represents tissue PDGF β receptor activation and reflects PDGF β receptor expression. Finally, the angiographic follow up varied from two to eight months, because patients who underwent earlier restenosis (< 4 months) had to be re-studied at that time for the evaluation of recurrent symptoms, and this could contribute to the variability of this study.

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