Pirfenidone Inhibits Early Myointimal Proliferation but has No Effect on Late Lesion Size in Rats

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Aims: intimal hyperplasia is mediated by smooth muscle cell proliferation, migration and deposition of extracellular matrix. The anti-fibrotic agent pirfenidone has been shown to inhibit pro-fibrotic growth factors in non-vascular inflammatory models. This study investigated the effect of the novel anti-fibrotic agent pirfenidone on the development of neointima.

Methods: male Sprague–Dawley rats received either standard diet or diet supplemented with pirfenidone (250, 500, 1000 mg/kg/day). Animals underwent left common carotid balloon angioplasty and were explanted at 4, 8, 14 and 28 days and analysed for intimal thickening, pro-fibrotic gene expression, extracellular matrix deposition and metalloproteinase activity.

Results: neointimal thickness was significantly reduced in a dose-dependent manner at 14 days; pirfenidone 250 mg/kg (p<0.005), pirfenidone 500 mg/kg (p<0.001), pirfenidone 1 g/kg (p<0.001). There were no significant differences in intimal thickening at 28 days. Expression of MMP-2, MMP-9, TIMP-1, collagen III and TGF-beta were all significantly inhibited at 14 days. Both collagen III expression and ECM deposition were reduced at 28 days (p<0.05 and <0.002 respectively).

Conclusion: pirfenidone reduces expression of MMPs governing smooth muscle cell proliferation and migration (MMP-2 and 9), and genes favouring ECM accumulation (TIMP-1 and collagen III). This study shows that inhibition of MMP activity is not sufficient to inhibit late lesion size.

Key Words: Intimal hyperplasia; Metalloproteinase; Extracellular matrix.

Introduction

Vascular smooth muscle cell (VSMCs) proliferation and migration along with the remodelling of extracellular matrix proteins (ECM) result in a hyperplastic intimal lesion. These are pathophysiological responses to a variety of stimuli including growth, hypertension and arterial injury. The formation of this “neointima” and subsequent luminal narrowing are responsible for the failure of arterial reconstructive procedures in bypass surgery,1 restenosis following transluminal angioplasty2 and arterial vasculopathy in transplanted grafts.3 Importantly, intimal hyperplasia predisposes to superimposed atherosclerotic changes, plaque rupture and vessel occlusion.4,5

Following mechanical or immune-mediated arterial injury macrophages, platelets and VSMCs release a myriad growth factors including platelet-derived growth factor (PDGF),6,7 basic fibroblast growth factor (bFGF)8,9 and transforming growth factor beta (TGF-β).10 These act as potent mitogens for medial VSMCs, inducing a phenotypic “switch” from a quiescent contractile state to a secretory proliferative form. This pivotal change is known as modulation.11,12 Medial VSMC proliferation and migration through the internal elastic lamina requires the degradation of ECM and subsequently synthesis and deposition of new extracellular proteins. Vascular smooth muscle cells are capable of producing metalloproteinases (MMPs) and serine proteases as well as ECM. MMPs are a family of Zn2+-dependent proteases that differ widely in substrate specificity and are not only capable of degrading matrix, but can also activate other metalloproteinases.13 Secretion of metalloproteinases, principally MMP-2 and 9 facilitate the migration of VSMCs across the internal elastic laminae, whilst TIMPs inhibit this process.14 Conversely, extracellular matrix deposition is characterised by a relative increase in protease inhibition.15 Inhibition of metalloproteinases therefore may offer a therapeutic strategy for the prevention of intimal hyperplasia and thus prolong saphenous vein

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material and methods

Arterial injury model

Four-month-old male Sprague-Dawley rats (weighing 350–400 g) were obtained from Harland (Cambridge, U.K.). Rats were anaesthetised with halothane and were cared for in accordance with Animals (Scientific Procedures) Act 1986. The left common carotid artery was denuded of endothelium with a saline-filled 2F balloon embolectomy catheter (Baxter Healthcare Corp) as described previously.\textsuperscript{19} In brief, the catheter was introduced through the left external carotid artery into the common carotid. Denudation was performed by inflating the catheter to a pressure of 2 atmospheres and passing it in a twisting manner from the arch of the aorta to the carotid bifurcation. This procedure was repeated twice more before the catheter was removed and the external carotid ligated. The rats were anaesthetised at 4, 8, 14 and 28 days post balloon injury and the left common carotid artery flushed with phosphate-buffered saline. The middle third of each vessel was used for histomorphometry and the outer thirds snap frozen in liquid nitrogen and stored at \( -70^\circ \text{C} \) for molecular studies.

Morphology

Vessels for histological study were not pressure perfused but fixed with 2.5% gluteraldehyde in 0.1 M phosphate buffer (pH 7.4). After 16 h further fixation, the vessels were embedded in epoxy resin and cut into 2 \( \mu \text{m} \) transverse sections. Intimal and medial areas were assessed using light microscope assisted computer planimetry. Intimal and medial areas were defined by internal and external elastic laminae respectively.

Sirius red staining

Carotid arteries were formalin fixed, embedded in epoxy resin and cut into 4 \( \mu \text{m} \) sections. The resin was removed in xylene for 10 min and dehydrated in serial washes of 100% alcohol for 2 min performed twice, 95%, 80% and 60% alcohol for 2 min. Sections were run under cold running water for 10 min and finally rinsed briefly with distilled water. Staining was performed overnight for 12 h in picro–sirius red F3BA (0.1% Sirius red F3BA in saturated aqueous picric acid). Rapid dehydration was repeated with an initial wash of 0.01 M HCl for 2 min, and serial washes of 70% alcohol for 45 s followed by 80%, 95% and 100% for 2 min. Slides were cleared with 2 washes of xylene for 2 min, excess xylene removed and slides mounted with Xam organic mountant. Ten random windows of intima were analysed and the value of extracellular matrix was determined from the mean of these windows.

Molecular analysis

The methods used to quantify levels of mRNA expression using reverse transcriptase–polymerase chain reaction (RT–PCR) have been described previously.\textsuperscript{20} In brief, vessels were cut into 10 \( \mu \text{m} \) sections and ground under liquid nitrogen in a mortar and pestle. Messenger RNA was extracted using oligo-dT-linked Dynabeads (Dynal, Bromborough, U.K.). Competitive reverse transcriptase–polymerase chain reaction (RT–PCR) was performed as described previously. Genes chosen for quantification in this study included matrix metalloproteinases (MMPs)-2 and 9, tissue inhibitor of metalloproteinases (TIMP-1), matrix protein collagen III and transforming growth factor-\( \beta \) (TGF-\( \beta \)). All probes and primers were designed from sequences available on the EMBL database using the program GCG prime (Genetics Computer Group, Madison, Wisconsin, U.S.A.) and synthesised by Life Technologies (Paisley, U.K.) or Genosys Biotechnologies Europe (Pampisford, U.K.). Quantification of RT–PCR products was performed using an enzyme-linked immunosorbent assay. Differences in tissue cellularity were corrected for by expressing values of complementary DNA product as a ratio to that of constitutively expressed housekeeping gene, \( \beta \)-actin.

Gelatin zymography

Metalloproteinases were extracted from frozen tissue as described previously.\textsuperscript{21} Briefly, frozen tissue was
thawed over ice, weighed, chopped into 1 mm² pieces and homogenised in buffer (urea 2 mM, Tris-HCl 50 mM pH 7.6, NaCl 137 mM, EDTA 1 g/l, Brij-35 1/ ml/l and PMSF 0.1 mM – all Sigma, Poole, (U.K.). The homogenate was then centrifuged at 12 000 g for 1 h at 4°C and the resulting supernatants dialysed (12–14 000 kDa) against a dialysing buffer (Tris 25 mM pH 8.5, CaCl₂ 10 mM, 0.1% Brij-35, 0.1 mM PMSF) for 18 h at 4°C. The protein concentration of each sample was standardised as described previously. Equivalent protein loads (15 µg) were resolved by a non-reducing electrophoresis through a 10% SDS-polyacrylamide gel impregnated with 1 mg/ml gelatin. The human fibrosarcoma cell line, HT-1080, was loaded onto each gel as a positive control. SDS was removed from the gels by incubation in 2.5% Triton X-100 (3 × 15 min) and the gels immersed for 16 h in incubation buffer (50 mM Tris-HCl, 10 mM CaCl₂ and 0.05% Brij-35, pH 7.4) at 37°C. Gels were fixed and stained in 0.1% Coumassie brilliant blue in 50% methanol/20% acetic acid/30% double-distilled water for 2 h. Proteinases were visualised as clear bands of lysis against a dark background of intact substrate. The relative density of each lytic band was determined from negative photographic images of gels with a Pharmacia LKB Imagemaster scanning densitometer (Pharmacia LKB, St. Albans, Herts, U.K.) and expressed as a product of the optical density and area of the band. This method provides a semi-quantitative analysis of metalloproteinase activity.

Statistical analysis

Intima medial ratios, collagen staining and RT–PCR data are presented as median ± range from 6 animals per group. Differences between control and treatment groups were calculated using non-parametric analysis (Mann–Whitney U-test). The relative density of substrate gel lysis was quantified from 3 carotids in each treatment group and run on the same gel. Differences were again expressed using non-parametric analysis. Differences were considered significant at ≤0.05.

Results

Stereological analysis

Pirfenidone inhibited intimal thickening at 14 days in a dose-dependent manner (p<0.001) (Fig. 1). Animals treated with pirfenidone at a dose of 1 g/kg/day showed 10% weight loss over this period compared to controls and thus pirfenidone was administered at a dose of 500 mg/kg/day throughout the remainder of the study.

“Uninjured” rat arteries do not contain smooth muscle cells in the intima. However, smooth muscle cells appeared in the intima of untreated rats 4 days post arterial injury, median intima medial ratio 0.08 (range 0.04–0.14) (Fig. 2A). Smooth muscle migration was completely inhibited by continuous daily oral administration of pirfenidone at 500 mg/kg/day (p<0.002) (Fig. 2). Intimal thickening was reduced in pirfenidone treated rats at day 8, intima medial ratio 0.38 (range 0.11–0.61) compared with controls, intima medial ratio 0.54 (range 0.26–0.93), p<0.01 (Figures 2C and D respectively). A significant reduction in intimal thickening was also observed at 14 days, intima medial ratio 0.49 (range 0.12–0.85) versus untreated rats, intima medial ratio 1.54 (range 1.02–2.04) p<0.001 (Figures 2E and F respectively). However, no significant differences in intimal thickening were observed at 28 days (Figures 2G, H and Figure 3). Pirfenidone had no significant effect on medial area compared with controls at any time point (data not shown).

Effect of pirfenidone on β-actin mRNA expression

RT–PCR was performed on carotid arteries from both control and treated animals to assess whether β-actin
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Fig. 2. Representative transverse cross-sections demonstrating the effect of pirfenidone (500 mg/kg) on the development of neointimal formation at 4 days (Control – A, Pirfenidone – B) 8 days (Control – C, Pirfenidone – D), 14 days (Control – E, Pirfenidone – F) and 28 days (Control – G, Pirfenidone – H). The arrow represents the internal elastic lamina (IEL).

Fig. 3. Bar graph showing intima medial ratios 4, 8, 14 and 28 days post arterial balloon injury. Intima media ratios significantly reduced in pirfenidone treated rats (500 mg/kg/day) at 14 days: p<0.001.

Fig. 4. Molecular data at 14 days: Values are expressed as medians with standard error of means shown. The value of messenger (mRNA) expression is stated in arbitrary units and expressed as a ratio to that of housekeeping gene β-actin.

- **a** MMP-2: P<0.001
- **b** MMP-9: P<0.001
- **c** TIMP-1: P<0.001
- **d** Collagen III: P<0.001
- **e** TGF-β: P<0.014

mRNA expression was affected by the administration of pirfenidone. No differences were found between control and treated animals at any time point assessed, confirming that expression of the house-keeping gene β-actin was not affected by either balloon injury or treatment with pirfenidone (data not shown).

**Effect of pirfenidone on pro-fibrotic gene expression**

The results at 14 days are shown in Figure 4 and expressed as mean values (±SEM). Gene data presented is standardised relative to the house-keeping gene β-actin. Pirfenidone significantly inhibited MMP-2, MMP-9, TIMP-1, TGF-β and collagen III expression at 14 days (p<0.001). These observations were not seen...
however at 28 days, when only collagen III mRNA was reduced in the Pirfenidone treated group, median value 0.87 (range 0.84–0.91) compared with controls, 1.12 (0.85–1.67) $p<0.05$.

**Metalloproteinase activity**

A representative gelatin zymogram is shown in Figure 6. Control samples demonstrated lytic bands at 92 kDa, 82 kDa and 72 kDa consistent with MMP-9, pro and active forms of MMP-2 respectively. Pirfenidone-treated samples also demonstrated clear lytic bands corresponding to pro and active MMP-2. However, densitometric analysis showed a significant reduction in MMP-9 activity in pirfenidone-treated carotids at day 4, median value 0.1 (range 0.02–0.2) compared with controls, 0.39 (0.27–0.63) $p<0.002$. Pro and active MMP-2 were reduced in pirfenidone-treated carotids at day 4 although this failed to reach statistical significance ($p<0.1$). MMP-9 activity was not detected at any other time point, consistent with previous findings. There were no significant differences in pro and active MMP-2 activity at any other time point.

**Extracellular matrix deposition**

Pirfenidone attenuated neointimal extracellular matrix deposition at 28 days; median value 15 (range 12–19) compared with controls, 9 (range 7–12) ($p<0.002$).

**Discussion**

This study shows pirfenidone to inhibit early vascular smooth muscle proliferation and migration following arterial injury but fails to inhibit late intimal proliferation at 28 days. Furthermore, the gene data presented suggests that its mechanism of action is due to inhibition of MMP-2 and 9 transcription. Gelatin zymography showed pirfenidone to significantly inhibit MMP-9 activity. Whilst the reduction in pro and active MMP-2 activity failed to reach statistical significance ($p<0.065$ and $p<0.093$ respectively), it is likely that pirfenidone also has an inhibitory effect on MMP-2 activation. Despite significant differences in intimal lesion size at day 14, continued administration of pirfenidone failed to confer this reduction in intimal thickening to 28 days. Additionally, pirfenidone had no effect on metalloproteinase expression at this later time point (Fig. 5).

Vascular smooth muscle cells constitutively express MMP-2 under basal conditions whilst MMP-9 expression is induced by arterial injury. It is therefore likely that the observed reduction in early intimal thickening is attributable to inhibition of MMP-2 and 9 in rats treated with pirfenidone. Previous studies using metalloproteinase inhibitors support this hypothesis: Southgate et al. showed synthetic MMP inhibitors significantly inhibited smooth muscle cell proliferation and migration from rabbit aortic explants in vivo and Bendeck et al. showed a significant reduction in intimal hyperplasia up to 10 days post rat
carotid ballon injury using the non-selective MMP-inhibitor GM-6001. Proliferation studies demonstrated a prolonged period of intimal vascular smooth muscle cell proliferation accounted for the accelerated increase in late lesion size. The reduction of TIMP-1 expression is more difficult to interpret since the role of TIMP-1 in the development of intimal lesions is complex. TIMP-1 is the major endogenous inhibitor of MMP activity, forming a 1:1 stoichiometric complex with MMPs, inhibiting both latent and activated enzyme. Following balloon injury, 10–30% of mediastinal smooth muscle cells proliferate within 24 h with migration to the neointima occurring by the third day. For proliferating SMCs to migrate to the neointima, cells must free themselves from their surrounding extracellular architecture. This is facilitated by a shift in the “balance of forces” between synthesis and degradation of ECM proteins towards degradation. Previous studies have shown TIMP-1 expression is detectable by 24 h following balloon injury but returns to baseline levels by day 7. We have shown pirfenidone to inhibit TIMP-1 production to 14 days. A reduction of TIMP-1 at this time point favours degradation at a critical time-point when intimal lesion size is increased solely due to the deposition of ECM proteins. Furthermore, we have shown both collagen III expression and deposition is synchronously inhibited by pirfenidone. Pirfenidone attenuates collagen deposition in many animal models of fibrosis and is currently under phase III trials for the treatment of idiopathic pulmonary fibrosis. Its effect in models of vascular disease has not been previously reported. Finally, TGF-β expression is suppressed in pirfenidone-treated rats. TGF-β is a polypeptide growth factor released by many different cell types facilitating cell proliferation, migration and augmenting ECM deposition in developing arteriosclerotic lesions. A reduction in TGF-β and subsequent decrease in collagen deposition has been previously observed in a bleomycin hamster model of fibrosis and this also occurred at a transcriptional level.

In conclusion, we have shown pirfenidone to attenuate early intimal thickening and significantly inhibit metalloproteinase gene expression governing medial smooth muscle cell proliferation and migration, and collagen deposition. The findings of this study support the hypothesis that metalloproteinases are pivotal mediators in vascular smooth muscle cell proliferation and migration. However we have shown that inhibition of metalloproteinases alone is not sufficient to inhibit late lesion formation and other pathways must be inhibited to address delayed migration and intimal proliferation.

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References


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