Migration of adventitial myofibroblasts following vascular balloon injury: insights from in vivo gene transfer to rat carotid arteries

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Abstract

**Objectives:** Migration of adventitial fibroblasts, in addition to smooth muscle cell proliferation, plays a role in neointima formation following vascular injury. Previous studies have not directly addressed whether endogenous adventitial cells migrate towards the intima following balloon injury in the absence of medial dissection. We have employed an in vivo gene transfer technique to the rat carotid artery to directly label adventitial fibroblasts prior to balloon injury. **Methods:** An adenoviral vector coordinating expression of nuclear targeted β-galactosidase (AdLacZ) suspended in pluronic gel was applied to the perivascular surface of left carotid arteries of male Sprague–Dawley rats. Balloon catheter mediated vascular injury was performed on these arteries 4 days later and animals killed at 3, 7 and 14 days after injury. **Results:** Expression of LacZ up to 14 days after application of the adenovirus was restricted only to the adventitia of uninjured arteries and absent from untransfected right carotid arteries. However, following balloon catheter injury, LacZ positive cells were observed within the medial layer of vessels by 3 days, and contributed to the population of cells within the neointima at 7–14 days. Adventitial cells in uninjured arteries did not express smooth muscle α-actin but after injury, LacZ positive cells migrating towards the lumen exhibited α-actin immunostaining, suggesting their change to a myofibroblastic phenotype. **Conclusions:** These findings provide direct evidence that adventitial fibroblasts migrate and contribute to neointima formation after balloon injury and show that in vivo gene transfer to the adventitia results in sustained transgene expression capable of labelling migrating adventitial cells within the media and neointima of injured vessels.

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**Keywords:** Restenosis; Remodelling; Angioplasty; Smooth muscle; Gene therapy

1. Introduction

Narrowing of the vessel lumen commonly occurs following percutaneous transluminal coronary angioplasty in humans and experimental vascular injury in animal models. Migratory and proliferative responses of cells within the vessel wall as well as deposition of extracellular matrix play key roles in restenosis and atherosclerosis [1]. Although medial smooth muscle cells (SMC) have been regarded as the main source of cells from which the neointima of injured vessels is formed through their luminal migration and proliferation [2], there is now increasing evidence that the adventitial layer can also be a significant contributor to the arterial remodelling process through increased angiogenesis [3], matrix deposition [4] and more importantly, fibroblast phenotypic transition to express SMC specific α-actin [5] and SM22 [6]. Furthermore, an earlier study addressing this issue showed that the neointima of injured canine carotid arteries consists of a heterogenous cell population and included myocytes that were identical to fibroblasts cultured from the adventitial surface [7].

Subsequent studies have suggested that these activated adventitial ‘myofibroblasts’ can migrate towards the lumen and contribute to the neointima of porcine coronary arteries following endoluminal injury [8,9]. However,
observations of adventitial cell migration towards the lumen in these reports have mainly been associated only with areas of severe balloon overstretch injury resulting in medial dissection and exposure of the adventitia to the vessel lumen. In addition, studies showing migration of adventitial cells in porcine coronary [8] and rat carotid [10] arteries have employed indirect techniques based on labelling cells in a replicative cycle with bromodeoxyuridine, which does not specifically identify cells solely of adventitial origin. Li et al. [11] have recently attempted to overcome this by showing that primary adventitial fibroblasts transduced in vitro, using retroviral particles to express β-galactosidase (LacZ), migrate in a luminal direction when reseeded onto a rat carotid artery following endoluminal injury. Although neointimal cells were reported to express β-galactosidase, these cells would have originated from those isolated, cultured and infected in vitro, a process likely to have induced phenotypic modulation of myofibroblasts [7,12] prior to their reintroduction in different animals. One recent study also employing the rat model has shown evidence contrary to the consensus that adventitial cells contribute to neointima formation [13]. In that study, the adventitia was labelled with a fluorescent dye at the time of balloon injury or 3 days later.

The present study employed an efficient in vivo gene transfer approach to directly demonstrate that endogenous adventitial cells do migrate towards the lumen following vascular injury. The perivascular arterial surface was transfected with the nuclear-targeted LacZ gene using an adenoviral vector suspended in pluronic gel prior to balloon injury. This approach was adopted since it has been reported to result in both efficient gene delivery [14,15] and minimal inflammatory responses [16]. In the absence of injury, β-galactosidase positive cells were restricted to the adventitial layer, however, following balloon injury, positive cells were observed in both the medial and neointimal compartments. These results now provide further evidence that adventitial fibroblasts can migrate and contribute to vascular remodelling following vascular balloon injury.

2. Methods

2.1. Animals

A rat carotid artery model was used to study the migration of adventitial fibroblasts after balloon injury. All studies were carried out under licensed approval of the Home Office, UK and carried out in accordance with guidelines of the University of Cambridge and UK government on animal welfare. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Male 3-month-old Sprague-Dawley rats (Charles River) weighing 380–450 g (n=10 per time point) were housed in pairs in a specific pathogen free environment and quarantined 10 days before use. For adventitial gene delivery and balloon injury of the left common carotid artery, animals were sedated with halothane (4% in O₂), anaesthetised with intraperitoneal administration of ketamine hydrochloride (Sigma, 72 mg/kg) and xylazine hydrochloride (Sigma, 5 mg/kg), and allowed to recover following all surgical procedures. Animals were euthanized under terminal anaesthesia at fixed experimental time points by exsanguination and retrograde aortic perfusion with 200 ml saline followed by 250 ml saline containing formalin (2% w/v) and glutaraldehyde (0.2% v/v) to fix the tissues before careful excision of the left common carotid artery and the contralateral vessel to avoid any damage to the adventitial layer.

2.2. Adventitial LacZ gene transfer and carotid artery balloon injury

A replication defective E1-deleted adenoviral vector coordinating nuclear-targeted expression of β-galactosidase (AdLacZ) was propagated in 293 cells, purified by cesium density ultracentrifugation and extensively dialysed before titration using a plaque assay as previously described [17]. To achieve efficient adventitial gene transfer, AdLacZ was suspended in pluronic F127 gel [14] (BASF, 25% w/v in sterile saline) maintained at 4°C, to obtain a final concentration of 1×10⁹ pfu/ml and kept on ice. The left common carotid artery of anaesthetised rats was isolated through a midline cervical incision to expose a 3-cm-segment of the artery from the bifurcation and 200 μl of AdLacZ containing pluronic gel applied to the perivascular surface of the artery. Following the rapid solidification of the gel around the artery at body temperature, the wound was closed and animals recovered. The same animals were anaesthetised 4 days later and the left carotid artery bifurcation re-exposed for balloon injury. A 2F Fogarty balloon catheter (Baxter) was introduced through the left external carotid artery and advanced 4 cm towards the thoracic aorta. The balloon was inflated with saline to distend the artery and was then pulled back to the bifurcation with constant rotation. This procedure was repeated two more times to ensure endothelial denudation and consistent vascular injury. After removal of the catheter, the external carotid artery was ligated, the wound closed and animals recovered. Rats were killed at 4, 7 or 14 days after LacZ gene transfer only or at 3, 7 or 14 days after balloon injury following the initial 4-day exposure to the adenovirus in pluronic gel. The right carotid artery served as a control and was not exposed to AdLacZ or injured.

2.3. Histological and immunocytochemical analyses

Following killing and perfusion of the rats, both left and right carotid arteries were fixed in saline containing
formaldehyde (2% v/v) and glutaraldehyde (0.2% v/v) for 24 h before washing in saline. In situ staining for LacZ was performed on 25-mm arterial segments using 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), the chromogenic substrate for β-galactosidase, for 18 h at 37 °C. These were then embedded in paraffin, sectioned (5 and 15 μm) and some sections were counterstained with nuclear fast red, haematoxylin and eosin, or Verhoeff-van Gieson stain for elastic tissues. Sections were deparaffinised with Histo-Clear (National Diagnostics) to minimise loss of the blue nuclear staining, indicative of cells expressing LacZ. For immunophenotyping cells, expression of smooth muscle α-actin was determined in sections incubated in 0.3% hydrogen peroxide for 30 min and blocked with 5% horse serum before using a primary monoclonal α-SM actin antibody (1:50, Clone 1A4, Dako) and secondary biotinylated horse anti-mouse antibody (1:200, Dako). Mouse IgG was used as a negative control instead of primary antibody. Positive immunostaining was determined using the Vectastain Elite ABC system (Vector Laboratories) and visualised with diaminobenzidine substrate (Sigma) followed by counterstaining with Harris haematoxylin (Sigma). To determine whether macrophages were present within the sections, a monoclonal primary antibody to CD68 (1:50, Clone PG-M1, Dako) was used. To ensure that residual LacZ adenovirus was not able to diffuse through the vessel wall after balloon injury, fixed arterial sections were subjected to immunofluorescent detection of adenoviral particles 24 h and 4 days after the addition of AdLacZ by use of a polyclonal FITC conjugated goat anti-adenoviral hexon antibody (1:50, Chemicon).

2.4. Quantitative measurements of cell migration and statistical analysis

A computer image analysis program (Adobe Photoshop 5.02) was used to quantify the proportion of adventitial cells contributing to the medial and neointimal compartments following balloon injury. Images of sections were captured into a PC using a CCD camera (Colorview 12, Soft Imaging System) attached to a microscope (Olympus BX51). The number of LacZ positive cells exhibiting blue nuclear staining was determined by using the image analysis software’s chromogen separation function as previously described [18]. The proportion of cells originating from the adventitia was estimated by determining the number of pixels exhibiting the blue chromogen in a given area. This number was divided by the average number of pixels per nucleus and expressed as a percentage of the total number of nuclei in the area determined by nuclear fast red staining. This estimate was carried out in six representative areas for each compartment of the vessel wall. The number of cells within the medial compartment was also counted at 0–3 days following balloon injury in LacZ transfected arteries. This was achieved by counting the number of black staining nuclei in haematoxylin and eosin stained sections. The medial compartment of each vessel wall was delineated and the image analysis software’s chromogen separation function used to identify stained nuclei. The number of pixels corresponding to stained nuclei was divided by an average number of pixels per nucleus, providing an estimate of the number of medial cells. This was done in four separate sections from four separate animals per time point. Results are reported as mean±S.E.M. and data compared using the unpaired Student t-test with P<0.05 considered as statistically significant.

3. Results

3.1. Adventitial LacZ gene transfer

Adventitial cells transfected with β-galactosidase exhibited blue stained nuclei after incubation with X-gal. Following pluronic gel mediated delivery of AdLacZ before balloon injury, LacZ positive cells were restricted solely to the adventitial compartment of left carotid arteries at 4, 7 and 14 days after gel application as shown in Fig. 1. In vivo transfection using the adenoviral vector alone to the adventitia did not result in any changes in morphology of the vessel wall up to 14 days after adenovirus delivery as evidenced by haematoxylin and eosin staining. Expression of LacZ was not observed at any time in the control right untransfected carotid arteries or in areas of left transfected arteries, which were not exposed to the pluronic gel (not shown). To maximise the LacZ transgene expression in the adventitia prior to balloon injury, a 4-day time period after gene transfer was chosen before balloon catheter injury was performed. To ensure that endogenous LacZ activity did not interfere with our observations, we confirmed that expression of LacZ was not observed at any time anywhere in control right untransfected carotid arteries or in areas of left transfected arteries, which were not exposed to the pluronic gel (not shown). LacZ gene expression was also not detected in untransfected arteries even after 14 days following balloon injury when tissues were subjected to X-gal staining. Immunohistochemical detection of adenoviral hexons in arterial sections at 4 h following transfection with AdLacZ revealed a high level positive immunofluorescent staining within the adventitial compartment only (Fig. 2A). However, at 24 h following transfection, staining is seen to diminish significantly (Fig. 2B), and at the time of balloon injury 4 days following transfection, no adenoviral hexons were detected in any compartment of the vessel wall (Fig. 2C). Combined with the lack of LacZ transgene expression observed in the medial compartment of uninjured vessels, this finding suggests that only adventitial cells were initially transfected and observed migrating towards the lumen following balloon injury. Non-transfected right carotid arteries did...
Fig. 1. Expression of β-galactosidase in the adventitial compartment of rat carotid arteries following in vivo LacZ gene transfer. Animals were sacrificed at 4 (A, D), 7 (B, E) or 14 (C, F) days after application of AdLacZ and sections stained with X-gal (A–C) or haematoxylin and eosin (D–F). Cells expressing LacZ exhibit blue stained nuclei and are confined to the adventitial (A) layer of arteries and not present in the media (M). Morphology of the vessel wall was unaltered by application of AdLacZ to the perivascular surface. Micrographs are representative of sections taken from 10 animals.

Fig. 2. Immunofluorescent localisation of adenoviral hexons following in vivo LacZ gene transfer. Animals were sacrificed at 4 h (A), 24 h (B, D) or 4 days (C) following application of adenovirus to left carotid arteries and immunostained with a FITC conjugated anti-adenoviral hexon antibody. A control right untransfected arterial section is depicted in panel D. Micrographs are representative of sections taken from four animals.
Fig. 3. Migration of adventitial cells towards the lumen was assessed at 3 (A, D), 7 (B, E) and 14 (C, F) days following balloon injury 4 days after the initial LacZ gene transfer. X-gal staining (A–C) shows several blue LacZ positive nuclei within the medial layer (M) of the artery 3 days after balloon injury, while at 7 days after injury blue stained nuclei are visible throughout the media and appear to migrate into the neointima (N) where by 14 days, these cells predominate. Haematoxylin and eosin staining (D–G) following balloon injury shows damage to the luminal surface at 3 days but the media remained intact (D). Initiation of neointima (N) formation occurs by 7 days (E), while a well developed neointima is present by 14 days (F) after the injury. The control uninjured right carotid artery is depicted in panel G. Micrographs are representative of sections taken from 10 animals.
Fig. 4. Staining of elastic tissues using Verhoeff–van Geison stain to demonstrate integrity of elastic laminae at (A) 3, (B) 7 and (C) 14 days following balloon injury. A, adventital; M, medial; and N, neointimal compartments are labelled. Micrographs are representative of sections taken from 10 animals.

Fig. 6. Expression of smooth muscle α-actin in rat carotid arteries following LacZ gene transfer. Application of AdLacZ did not induce expression of α-actin in the adventitial (A) layer of arteries and positive staining (brown) was restricted to the media (M) at 4 (A), 7 (B) or 14 (C) days after gene transfer, determined by immunostaining with a monoclonal antibody to α-actin. LacZ positive cells which do not exhibit α-actin staining are present in the adventitia layer. Micrographs are representative of sections taken from 10 animals.

Fig. 7. Expression of smooth muscle α-actin in rat carotid arteries following LacZ gene transfer and balloon injury. Cells exhibiting both positive smooth muscle α-actin immunostaining (brown) and LacZ X-gal staining (blue) are evident in the medial (M) and neointimal (N) layers at 7 (A) and 14 (B) days after vascular injury. Arrows denote cells co-stained with α-actin and X-gal in the neointimal layers. Micrographs are representative of sections taken from 10 animals.
not exhibit any positive adenoviral hexon immunostaining (Fig. 2D).

### 3.2. Migration of transfected adventitial cells

Following balloon injury of transfected left carotid arteries, some cells exhibiting nuclear LacZ staining were observed in the medial layer of the vessel wall at 3 days and by 7 days these had migrated further towards and into the neointima, whilst a pool of LacZ positive cells remained within the adventitia (Fig. 3A,B). The neointima was well developed by 14 days after injury and contained a large proportion of cells showing positive LacZ staining (Fig. 3C). Changes in vessel wall morphology and neointima formation following injury are shown by haematoxylin and eosin staining in Fig. 3D–G. Changes in the luminal surface are apparent by 3 days after injury with the medial layer remaining intact. Characteristic neointimal formation was present at 7 days and well developed by 14 days, with no continuum between adventitia and neointima observed in any arteries, as shown by Verhoeff-van Gieson staining in Fig. 4 depicting intact elastic laminae. The increases in the presence of transfected adventitial cells in the medial and neointimal compartments were analysed using a computer Photoshop software package. As summarised in Fig. 5, the percentage of LacZ positive nuclei increased in the media from 3 to 7 days and in the neointima from 7 to 14 days, while medial and adventitial expression of LacZ positive nuclei decreased over the latter time period. The number of cells in the medial compartment significantly decreased immediately and 1 day following balloon injury, compared with the number estimated in the media of control uninjured arteries as shown in Table 1. However, no areas of the media of balloon injured arteries were devoid of cells at any time. Medial cell number returned towards control levels by 72 h following the vascular injury.

### 3.3. Phenotypic modulation of adventitial cells

In the absence of vascular injury, expression of smooth muscle α-actin was restricted to the medial layer of the vessel wall for up to 14 days following adventitial delivery of AdLacZ only (Fig. 6). However, at 7 and 14 days following balloon injury, a few cells within the adventitia exhibited positive immunostaining for smooth muscle α-actin, while LacZ positive myofibroblasts which had migrated from the adventitia into the medial and neointimal compartments expressed α-actin (Fig. 7), suggesting a modulation in adventitial cell phenotype induced by the balloon injury. At no time, whether in the presence or absence of balloon injury, were macrophages observed within the vessel wall following LacZ gene delivery determined by macrophage CD68 immunostaining (data not shown).

### 4. Discussion

The nature and origin of cells which constitute the neointima of atherosclerotic and restenotic lesions has been widely debated over the past decade [19]. The present study now provides further evidence that adventitial fibroblasts can migrate towards the lumen following vascular endoluminal injury and take on a myofibroblastic

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Table 1

<table>
<thead>
<tr>
<th>Days following balloon injury</th>
<th>Control</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial cell number per cross-section area</td>
<td>536±67</td>
<td>256±72*</td>
<td>359±62*</td>
<td>429±49</td>
<td>567±58</td>
</tr>
</tbody>
</table>

Values are expressed as mean±S.E.M. (n=6), *P<0.05 compared to control uninjured arteries. The number of cells within the medial compartment was counted at 0–3 days following balloon injury in LacZ transfected arteries using image analysis software as described in Methods.

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![Fig. 5. Quantification of adventitial cell migration 3–14 days following balloon injury. Computer image analysis was used to determine the percentage of LacZ expressing nuclei exhibiting the blue chromogen and expressed as an average percentage of positive nuclei compared to the total number of nuclei in six representative areas in each compartment. Data representative of 10 animals in each treatment group. Mean±S.E.M., ★P<0.05 compared to uninjured arteries (day 0). +P<0.05 compared to values at 3 days following injury, †P<0.05 compared to neointima at 7 days following injury.](image-url)
phenotype. Tracking the migration of endogenous adventitial fibroblasts by in vivo vascular gene transfer prior to balloon injury, which did not result in medial dissection, has enabled us to employ a novel approach to complement other studies in the literature [8,11]. In particular, we have shown that endogenous adventitial cells transfected in vivo remain static in the absence of balloon injury but can migrate into the medial and neointimal layers following vascular injury, which did not involve severe medial disruption where a continuum exists between the adventitia and lumen. Furthermore, cell labelling with LacZ by gene transfer does not rely on cell metabolic properties unlike BrdU techniques previously used [8], which only labels proliferating cells at the time of administration and thus is not specific to adventitial cells. Co-localisation of cells expressing smooth muscle α-actin and exhibiting nuclear LacZ staining in the vessel wall following injury suggests that adventitial cells underwent a phenotypic transition to myofibroblasts when they migrated. There is no histological evidence in the present study to suggest that the adventitia was damaged following adeno viral gene delivery. Expression of smooth muscle α-actin was unaltered up to 14 days following application of the adenovirus alone and no migration of any LacZ positive cells into the media was observed in the absence of balloon injury. Nevertheless, we acknowledge that the possibility exists that gene transfer per se prior to balloon injury may have affected the adventitial cells. In addition, despite the loss of medial cell number following balloon injury, complete loss of medial cells or rupture of the medial compartment was not observed in any balloon injured arterial sections in this study.

The diminution of LacZ transgene expression in the present study due to dilution of episomal DNA over time and loss of the blue X-gal chromogen staining by histological processing techniques would have led to an underestimation of adventitial cell migration and their contribution to neointima formation. Nevertheless, we have demonstrated that a significant proportion of LacZ labelled adventitial myofibroblasts remain in the media and neointima at 14 days after balloon injury. In the report by Li et al. [11], similar adventitial cell migration after vascular injury was shown using extrinsic fibroblasts transfected with LacZ in vitro, however, the possibility cannot be excluded that these cells may have undergone phenotypic modulation [7,12] under culture conditions prior to their reseeding on the arterial perivascular surface, thereby making them more likely to migrate. The findings of our study differ from those of De Leon et al. [13] where adventitial cells were not observed to migrate following balloon injury. This may have resulted from differences in the nature and timing of the label used. Nevertheless, our study is entirely consistent with the consensus that adventitial cells contribute to neointima formation and vascular remodelling [20]. Therefore, the in vivo labelling of adventitial fibroblasts prior to balloon injury with a long lasting marker gene in our present study represents an efficient and sustained method of tracking the migration and phenotypic modulation of these cells after vascular injury and provides evidence consistent with the consensus that adventitial cells contribute to neointima formation and vascular remodelling [20].

Neointima formation has been thought to arise from migration and proliferation of medial SMC under the influence of cytokines including platelet-derived growth factor [21], transforming growth factor-β [22] (TGF-β1) and basic fibroblast growth factor [23]. However, human restenotic specimens have been shown to contain very low amounts of proliferating cell nuclear antigen and histone 3 mRNA suggesting that redistribution of components within the vessel wall [24], such as adventitial cell migration may contribute to a greater extent to neointima formation than medial and intimal SMC proliferation. It is likely that phenotypic modulation of medial SMC occurs in addition following arterial injury. Following angioplasty which resulted in complete medial rupture in porcine coronary arteries, Christen et al. [25] showed that the majority of neointimal cells expressed markers of late SMC differentiation and suggested that myofibroblasts could also conceivably undergo complete phenotypic modulation to a mature differentiated SMC phenotype. Furthermore, it is likely that vascular injury induces SMC movement towards the lumen ahead of a wave of adventitial cell migration. Our observations are consistent with this possibility since labelled adventitial cells are initially only observed in the media and on the periphery of the neointimal compartment at 7 days following balloon injury, while by 14 days labelled cells had infiltrated into the neointima, however, the neointima could also contain adventitial cells which have lost their LacZ expression.

Recent evidence has added weight to the hypothesis that adventitial cell migration can contribute to vascular remodelling [19]. TGFβ-1, in particular, has been reported to play a role in adventitial cell phenotype modulation [26], and induction of matrix metalloproteinases and their tissue inhibitors can regulate adventitial remodelling and myofibroblast motility [27] after vascular injury. In addition, perivascular inflammation arising from adventitial accumulation of neutrophils and macrophages following balloon angioplasty [28] may contribute to increased generation of reactive oxygen species and cytokines within the adventitial compartment and thus contribute to growth factor release and facilitation of fibroblast phenotypic transition, proliferation and migration [29–32]. It is possible that the initial loss of medial SMC observed following vascular balloon injury occurs via apoptotic cell death as previously reported by Pollman et al. [29]. Initial repopulation of the medial compartment has been previously characterised to occur by rapid medial smooth muscle cell proliferation [2], followed by migration of adventitial myofibroblasts towards the lumen as shown in this study and by others. It has been reported that increases in expression of matrix
metalloproteinases following vascular balloon injury may facilitate the migration of adventitial cells through the vessel wall to populate the neointima [33], however, it remains to be elucidated whether similar mechanisms contribute to the extensive migration of adventitial cells towards the neointima observed in the present report.

Our present findings that adventitial fibroblasts contribute to vascular remodelling and that transgene expression can be maintained in the adventitia and detected in the media and neointima following injury, therefore provides further direct evidence that adventitial gene transfer can be an efficient approach to investigate mechanisms by which adventitial fibroblasts are activated to migrate following balloon angioplasty, and to develop future strategies for vascular gene therapy.

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