Direct In Vivo Evidence Demonstrating Neointimal Migration of Adventitial Fibroblasts After Balloon Injury of Rat Carotid Arteries

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Background—Clinical and experimental evidence suggest that the adventitia participates in the response to endoluminal vascular injury. The current study used a direct approach to test the hypothesis that, after balloon injury of the rat carotid artery, adventitial fibroblasts migrate in a luminal direction and contribute to neointima formation.

Methods and Results—Primary syngeneic adventitial fibroblasts were stably transduced with retroviral particles coordinating expression of β-galactosidase (LacZ) and introduced into the adventitia of right carotid arteries of rats immediately after balloon injury. At defined times after injury and fibroblast implantation, rats were euthanized, and arterial tissue was examined for detection of LacZ mRNA (reverse transcription polymerase chain reaction), DNA (polymerase chain reaction), and in situ enzymatic activity. LacZ expression was detected in the media 5 days postinjury and in both media and neointima at 7, 10, and 14 days postinjury. LacZ was undetectable in injured vessels that had not been seeded with transduced fibroblasts and was restricted to the adventitia in seeded vessels that were not injured.

Conclusions—These observations provide direct demonstration of adventitial fibroblast migration into neointima of arteries after endoluminal injury. (Circulation. 2000;101:1362-1365.)

Key Words: adventitial fibroblasts ▪ migration ▪ carotid arteries ▪ vascular injury

Adventitial activation has been reported in coronary arteries of victims of fatal coronary artery disease and in some individuals that appeared to antedate intimal disease.1,2 Neointima formation also has been observed in response to adventitial injury in various animal models.3,4 Further, endoluminal injury of the porcine coronary artery has been shown to result in significant remodeling of the adventitia, characterized by proliferation of adventitial fibroblasts.5–6 Similarly, BrdU labeling studies7 have demonstrated increased adventitial proliferation within 3 days after endoluminal balloon injury of the rat carotid artery, which progressed over time to the neointimal compartment.

These findings provided indirect evidence for participation of adventitial cells in neointima formation after endoluminal vascular injury, because BrdU cannot selectively identify specific cells of adventitial origin. The inability to identify cells that entered the replicative cycle before or after BrdU administration and decreasing intensity of BrdU staining with time, as a result of the dilutional effect of ongoing cell division, makes it difficult to use this technique over prolonged periods.8

The current study used a more direct approach to test the hypothesis that adventitial fibroblasts migrate in a luminal direction into the neointima after endoluminal vascular injury. Syngeneic fibroblasts, derived from the adventitia of rat carotid arteries, were stably transduced with a β-galactosidase reporter gene and introduced into the adventitia of rat carotid arteries immediately after balloon injury. Results suggest that endoluminal injury of the rat carotid artery induces the migration of fibroblasts from the adventitia, through the medial layer, and into the neointimal compartment.

Methods

Syngeneic Adventitial Fibroblasts

Primary cultures of adventitial fibroblasts9 were recovered from the carotid arteries of female Sprague-Dawley rats (Charles River, Wilmington, Massachusetts), transduced with retroviral particles encoding β-galactosidase (LacZ), isolated by fluorescence-activated cell sorting, and expanded in complete media (Dulbecco’s Modified Eagle Medium [DMEM] containing 10%, vol/vol, fetal bovine serum, 4 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin).

Animals

Female Sprague-Dawley rats (n=4/5 per time point) were subjected to ovariectomy 3 days before balloon injury of the right carotid artery.7 The left carotid artery was subjected to the same dissection procedure but was not injured. Transduced adventitial fibroblasts...
(7×10^3 cells) were introduced into the adventitia of both the injured right and uninjured left carotid artery immediately after injury. Additional controls included the addition of vehicle only (DMEM) into the adventitia of each carotid artery.

**Biochemical Analyses**

At defined times after injury and fibroblast implantation, rats were euthanized. Both carotid arteries were recovered, stained (6 hours, 30°C) with X-Gal, fixed (24 hours) in 10% (vol/vol) formalin, embedded in paraffin, thin-sectioned (5 μm), counterstained with nuclear fast red, and examined for characteristic blue staining of LacZ enzymatic activity.

Neointima and media of carotid arteries 14 days after injury were harvested, and neointima pooled from 5 animals was incubated (45 minutes, 27°C) in 4 mL of dispersing medium (1 mg/mL collagenase, 0.1 mg/mL elastase, 0.5 mg/mL soybean trypsin inhibitor, 1 mg/mL bovine serum albumin, 200 U/mL penicillin, and 200 μg/mL streptomycin in DMEM). Isolated neointimal cells were collected through a nylon mesh filter, resuspended, and expanded to confluency in tissue culture using complete media. DNA and RNA were extracted from medial and neointimal explant pools, as well as primary cultures of transduced fibroblasts and neointimal cells. Total genomic DNA (0.1 μg) or RNA (1.0 μg) was used in polymerase chain reaction (PCR) (DNA) and reverse transcription polymerase chain reaction (RT-PCR) (RNA) assays under previously established conditions with defined DNA amplimer sequences specific for rat glyceraldehyde-3-phosphate dehydrogenase mRNA and the LacZ transgene.

**Results**

The right carotid arteries were examined histologically at 5, 7, 10, and 14 days after balloon injury and adventitial implantation of transduced fibroblasts (Figure 1). Circumferentially uniform layers of neointimal cells first appeared at 7 days, and this neointima compartment gradually increased in thickness from days 7 to 14. The time course and extent of neointima formation were similar, regardless of whether arteries were seeded with transduced fibroblasts or vehicle alone. The time course of this injury response was consistent with previous efforts and suggested that adventitial implantation of transduced fibroblasts did not alter the kinetics of neointima formation. Within 5 days of injury, LacZ-positive fibroblasts (in situ enzymatic activity) were readily detected in the adventitia and consistently observed in the medial compartment (Figure 1A). Over the time interval of 7 to 14 days postinjury, LacZ-positive staining was readily apparent in the adventitia and consistently observed in the media compartment (Figure 1D). Neointima was undetectable in the left uninjured carotid arteries at all time points, including 14 days posttreatment (Figure 1E). Positive staining for LacZ-transduced fibroblasts initially was localized to the adventitia and gradually disappeared over the next 14 days (Figure 1E). No LacZ staining was observed at any time point either in the media compart-
ment of uninjured vessels or in vessels not implanted with transduced fibroblasts (Figure 1F).

A characteristic RT-PCR product (355 bp) was identified for LacZ mRNA in both transduced fibroblasts (Figure 2A) and expanded populations of neointimal cells recovered from vessels after injury and implantation of LacZ-positive fibroblasts (Figure 2B). LacZ mRNA and DNA were restricted to medial (Figure 2C) and neointimal (Figure 2D) compartments of balloon injured vessels (14 days posttreatment) that had been seeded with transduced fibroblasts and were undetectable in unseeded injured vessels (Figures 2C and 2D).

**Discussion**

In the rat carotid artery, the response to endoluminal balloon injury begins with destruction of the endothelium and compression damage to medial vascular smooth muscle cells (VSMCs). The extent of medial injury induces the production of growth factors, cytokines, chemoattracrants, and reactive nitrogen/oxygen species that may play a regulatory role in both promoting early adventitial activation and determining the final extent of neointima formation. Furthermore, cytokine induction of matrix metalloproteinases and their tissue inhibitors after vascular injury predict their involvement in regulating adventitial remodeling and fibroblast motility. Indeed, our previous studies determined that media conditioned by activated VSMCs in vitro induced the migration of well-characterized adventitial fibroblasts, including those stably transduced with LacZ. Consequently, we anticipated that the release of factors from VSMCs damaged in vivo would stimulate adventitial fibroblast activation and migration, thereby promoting their participation in the injury response.

The approach of studies presented in the current study permitted unequivocal assessment of both the response of a distinct cell type of known origin to endoluminal injury and its direct contribution to neointima formation. Because there are no specific markers to distinguish fibroblasts from VSMCs and other undifferentiated cell types, harvested adventitial fibroblasts were transduced with a LacZ reporter gene. Implantation of LacZ-positive fibroblasts into the adventitia of carotid arteries provided a new interventional strategy that qualitatively confirmed the contribution of these cells to endoluminal vascular injury.

The appearance of LacZ-positive fibroblasts within the neointima is consistent with mounting indirect experimental results, suggesting that neointima formation includes the involvement of the adventitia. However, the ability to quantify the extent of this involvement under the current experimental design is limited by the following considerations: (1) The intensity of in situ LacZ staining appeared to coincide with previously identified (BrdU) areas of increased proliferation, an observation suggesting that the transcriptional activity of the retroviral promoter may be cell-cycle dependent. Consequently, LacZ mRNA and enzymatic activity may not correlate with fibroblast number. Because retroviral transduction results in integration of a single copy of the transgene, PCR (DNA) quantitation of marker gene copy number should correlate with transduced cell number independent of cell cycle status. (2) Competition between seeded transduced cells and intrinsic adventitial fibroblasts for limited growth factors, cytokines, and other factors released from the injured vessel may result in an underestimation of the adventitial contribution to neointima formation. Seeding of transduced fibroblasts after balloon injury and removal of the native adventitia may provide a more quantitative assessment of the adventitial response. Whether these approaches provide a means to quantitate the injury response, to probe the involved cellular and molecular mechanisms, and to develop a rational therapeutic strategy remains to be determined.

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