The fetal origin of adult atherosclerosis : a study in ApoE and Ldlr mouse models

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Chapter 5

Endothelial-to-Mesenchymal-Transformation during the Initial Stages of Neointima Formation

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Abstract

In a previous study we demonstrated that embryonic endothelial cells in vivo have the capacity to undergo endothelial-to-mesenchymal-transformation (EMT). We hypothesized that adult endothelial cells also have that capacity in vivo during the initial phase of neointimal lesion development. To investigate this, we induced neointimal lesions, a reflection of cardiovascular disease, in Tie2LacZ mice by placement of a nonconstrictive collar around the femoral artery. Three days after collar placement we detected endothelial detachment and proliferation. In addition, a decrease in phosphoSmad2 staining, a Tgf-β-related protein associated with differentiation and quiescence, was observed. From 7 days onwards β-galactosidase positive mesenchymal cells were found in the developing neointimal lesions. A number of these β-galactosidase-positive mesenchymal cells also showed positivity for α-smooth muscle actin. Since Tie2 is also expressed in part of the bone marrow cells, we assessed their contribution to neointimal lesion formation. Beta-galactosidase-positive bone marrow-derived cells were recruited to the adventitia, but could not be detected in the neointima and media. We conclude that adult vascular endothelial cells in vivo have the capacity to undergo EMT. Furthermore, through EMT, endothelial cells contributed to the total pool of mesenchymal cells that compose the neointima. These data indicate that EMT could be important in development and progression of cardiovascular disease.

Introduction

In a previous study using a quail model, we demonstrated that embryonic endothelial cells in vivo have the capacity to undergo endothelial-to-mesenchymal-transformation (EMT).[1] During the initial phases of arterial differentiation, embryonic endothelial cells of the descending aorta detached from the epithelial layer and differentiated into subendothelial mesenchymal cells. The transformation from endothelial to mesenchymal phenotype was accompanied by a reduction in the expression of the endothelial marker QH1 and gain of α-smooth muscle actin positivity. A number of reports substantiated our data and appointed a role for Tgf-β signaling in this process.[2-4] However, it remained unclear whether also adult endothelial cells have the potential to transdifferentiate into mesenchymal cells in vivo.

Therefore, in this study we investigated the capacity of adult vascular endothelial cells to undergo EMT in vivo. For this purpose we used Tie2LacZ reporter mice.[5] The Tie2 gene encodes a vascular endothelial-specific receptor.[6,7] Subsequently, in Tie2LacZ reporter mice positivity for β-galactosidase in the normal vascular wall is exclusively found in the endothelial cell population. Adult vascular endothelial cells are fully differentiated and are in a state of quiescence. To study the capacity of these cells to undergo EMT, we challenged the mouse vascular system by placement of a nonconstrictive collar around the femoral artery. This technique is frequently used to induce neointimal lesions, a reflection of cardiovascular disease.[8-12] Besides determination of the EMT capacity of adult endothelial cells in vivo, this experimental set-up enabled us to assess whether endothelial cells are active contributors to the mesenchymal cell population in neointimal lesions.

To investigate the role of Tgf-β signaling in our model, the expression patterns of several proteins of the Tgf-β/Smad signal transduction pathway[13] were studied. Tgf-β can signal via two different pathways on endothelial cells. Tgf-β signaling through the type I receptor Alk5 and Smad2/3 has been reported to maintain a quiescent differentiated phenotype of the endothelium.[14] Signaling through the Tgf-β type I receptor Alk1 and Endoglin via Smad1/5/8 on the other hand, stimulates proliferation and migration of endothelial cells.[15]

Our results demonstrate that adult endothelial cells have the capacity to undergo EMT in vivo. Furthermore, we show that a first population of mesenchymal cells in neointimal lesions is of endothelial cell origin.

Methods

Mice

For these experiments we used Tie2LacZ, Rosa26LacZ and wild-type FVB mice. The Tie2LacZ reporter line (a generous gift of Dr. U. Deutsch) was derived from founder 182.30 as described.[5] The construct contained the 2.1 kb *HindIII* Tie2 promoter fragment followed by the LacZ reporter gene and simian virus 40 poly(A) signal sequence. In addition, a mouse Tie2 genomic fragment extended from an NgoMI site at the 3' end of exon 1 approximately 10 kb into the first intron downstream of the LacZ cassette (HHNS construct). The founders were backcrossed to FVB for more than 10 generations. The Rosa26LacZ reporter mouse, a knock-in mouse expressing the LacZ gene in essentially all tissues, was kindly provided by Dr. P. Soriano.[16] Wild-type FVB mice were generated by our internal breeding facility. Diet and water were provided ad libitum. The Committees on Animal Welfare, Leiden University Medical Center and TNO Quality of Life, approved all animal experiments.

Femoral Artery Collar Placement

In Tie2LacZ mice average age 6 months, a nonconstrictive collar was placed around the femoral artery to induce neointima formation.[8,9] With this technique neointimal lesions induced within the area of the collar. The mice were anesthetized by an intraperitoneal injection of 2.5 µl/g Hypnorm® (containing fentanyl citrate 0.315 mg/mL and fluanisone 10 mg/mL, Bayer, Mijdrecht, The Netherlands) and 2.5 µl/g Dormicum® (midazolam 5 mg/mL, Roche, Woerden, The Netherlands). The nonconstrictive polyethylene collar (0.40 mm inner diameter, 0.80 mm outer diameter, 1.5 mm length, Portex, Hythe, UK) was placed around one of the femoral arteries avoiding damage to the vascular wall. The other hind leg was sham-operated.

Beta-Galactosidase Staining

Tie2LacZ mice were sacrificed at 3, 7, 14 and 21 days (n=7, 16, 27 and 4) and the bone marrow recipients 14 days after collar placement, respectively. After cervical dislocation both collared and sham-operated femoral arteries were harvested. For detection of the β-galactosidase by light microscopy, tissues were first fixed in 2% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 2 hours at 4^{o}C followed by incubation for 2 hours at 37 ^{o}C in a solution containing 10 mM potassium ferricyanide, 10 mM potassium ferrocyanide, 2 mM $MgCl_2$ and 0.1%

X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, Roche). Postfixation was performed overnight in 4% PFA after which the tissue was dehydrated in series of ethanol and xylene, and paraffin embedded.

For visualization of the β-galactosidase staining reaction by transmission electron microscopy the arteries were fixed in 2% PFA/0.1% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 1 hour at 4 ^{o}C followed by incubation in a solution containing 10 mM potassium ferricyanide, 10 mM potassium ferrocyanide, 2 mM $MgCl_2$ and 0.1% Bluo-gal (5-bromo-3-indolyl-beta-D-galactosidase, Invitrogen, Breda, The Netherlands) for 2 hours at 37 ^{o}C. Postfixation was carried out for 12 hours in 2% PFA/2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) and in 1% OsO_4/PBS for 1 hour. The tissue was embedded in epoxy resin mixture and sections of 1 µm were cut. Sections of interest were re-embedded, ultra thin-sectioned and subsequently stained with lead citrate and uranyl acetate. The EM410 transmission electron microscope (Philips, Eindhoven, The Netherlands) was used for assessment of the β-galactosidase staining.

Immunohistochemistry

Overnight fixation of the tissues in 2% acetic acid in 98% ethanol or in 3% PFA in 66% ethanol was followed by dehydration in series of ethanol and xylene and the tissues were paraffin embedded. Transverse 5-µm sections were cut and serially mounted. Unless indicated otherwise the immunohistochemistry was performed as previously described.[17] Sections were incubated overnight at room temperature with rat monoclonal antibodies against ICAM-1 (1:100, R&D systems, Abingdon, UK), CD31 (1:500, Serotec, Düsseldorf, Germany), CD34 (1:50, PharMingen, Breda, The Netherlands), and Mac-3 (1:400, PharMingen). The rabbit polyclonal antibodies against phosphoSmad1/5/8 (PSmad1/5/8; 1:50) and phosphoSmad2 (PSmad2; 1:1500) were generated as previously described.[18] Furthermore, rabbit polyclonal antibody against Von Willebrand factor (vWF; 1:500, DAKO, Glostrup, Denmark) and goat polyclonal antibodies against VCAM-1 (1:200, Santa Cruz, Heerhugowaard, The Netherlands) and Ki67 (1:1000, Sanver Tech, Heerhugowaard, The Netherlands) were used. Rabbit anti-rat biotin conjugate (1:200, Vector, Amsterdam, The Netherlands), goat anti-rabbit biotin conjugate (1:200, Vector) or rabbit anti-goat biotin conjugate (1:200, Vector) with normal rabbit and goat serum diluted in PBS were used as secondary antibodies and incubated for 1 hour at room temperature. Biotin labeling was followed by incubation with Vectastain ABC (Vector) for 1 hour. 3-3' diaminobenzidine tetrahydrochloride was used for visualization and counterstaining was performed with Mayer's hematoxylin.

For staining of the sections with mouse monoclonal anti-α smooth muscle actin (1:2000, Sigma-Aldrich), the primary antibody was coupled overnight to polyclonal rabbit anti-mouse peroxidase conjugate (1:200, DAKO) secondary antibody.[19] Sections were incubated for 2 hours at room temperature. Several X-gal sections were stained with nuclear red using the standard protocol.

Bone marrow transplantations

To demonstrate that the neointimal cells expressing X-gal, Bluo-gal and α-smooth muscle actin were not derived from circulating cells expressing Tie2, the bone marrow of wild-type mice was replaced by bone marrow of Tie2LacZ and Rosa26LacZ mice and followed by collar placement 4 weeks after transplantation.

The drinking water of wild-type bone marrow recipients (n=8) was supplemented with the antibiotics polymixin B (Bupha, Uitgeest, The Netherlands), ciprofloxacin (Bayer), amfotericine B (Bristol-Myers Squibb, Woerden, The Netherlands) and saccharose from week -1 until +4 after bone marrow transplantation. Donor bone marrow was harvested from femurs and tibias of Tie2LacZ (n=4) and Rosa26LacZ (n=4) mice. Bone marrow recipients were irradiated with a single dose of 13 Gy X-ray total body irradiation as previously described.[20] One day after irradiation approximately $1x10^7$ donor cells were injected via caudal vein injection.

Statistical analysis

The endothelial cell proliferation response was studied in femoral arteries 3 days after collar placement or sham-operation (n=6 each group). The total number of nuclei and the total number of Ki67-positive nuclei were determined by counting the nuclei in each fifth section of 5 μm over a length of 0.7 mm. The lumen volume was estimated by applying Cavalieri's principle[21] and used to calculate and correct for endothelial surface area. Data are represented as mean ± SEM. Statistical significance of the differences between the groups were determined using the Student's *t*-test and considered significant if $P < 0.05$.

Results

Time Course of Neointima Formation

We studied the neointimal response at 3, 7, 14 and 21 days after collar placement and sham-operation. Normal vascular morphology was observed in the femoral arteries of sham-operated mice at all time points after intervention (Figure 1A). Similar morphology was detected in areas outside the region of the collar. Within the collar region on the other hand, 3 days after collar placement an endothelial cell response was observed characterized by detachment and proliferation (Figure 1B). Morphometric analysis based on the Ki67 staining revealed a significantly increased proliferation rate of the endothelial cells in collared femoral arteries compared with sham-operated femoral arteries (35.05 ± 6.09 versus 1.44 ± 0.61% Ki67-positive cells, $P < 0.001$, Figure 1C).

One layer of subendothelial cells was present after 7 days and 2 to 3 layers could be detected 14 days after collar placement. Endothelial cells of both collared and sham-operated femoral arteries showed positivity for vWF (Figure 2A-B). In addition to positivity in the endothelial cell lining, vWF deposition was found in the neointima of collared femoral arteries (Figure 2B). ICAM-1 expression in endothelial cells of collared femoral arteries was decreased compared with sham-operated arteries (Figure 2C-D). The CD31 staining remained unaffected and was restricted to the endothelial cells throughout the process of neointima development (Figure 2E-F). From 14 days onwards the first signs of fragmentation of the internal elastic lamina were observed and medial smooth muscle cells started to migrate into the neointima. After 21 days large parts of the internal elastic lamina had disappeared and many smooth muscle cells were present in the neointima (data not shown).

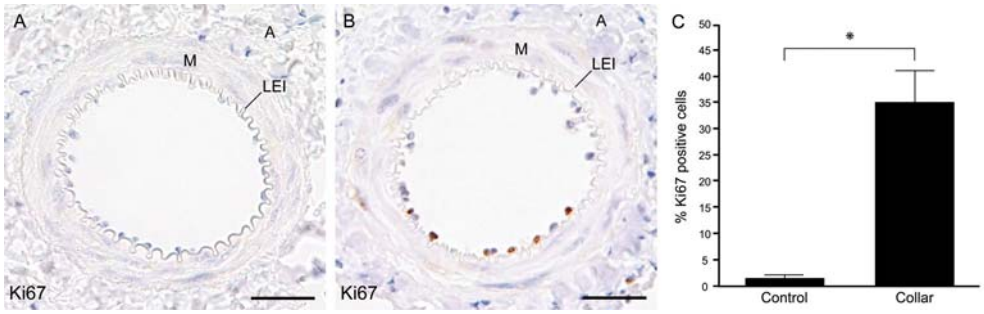

Figure 1. Endothelial cell proliferation 3 days after collar placement. Representative immunostaining for Ki67 in (A) sham-operated and (B) collared femoral arteries. LEI indicates lamina elastica interna; M, media; A, adventitia. Scale bar: 30 µm. (C) Morphometric analysis of endothelial cell proliferation in the region of the collar compared with sham-operated femoral arteries. Data are means ± SEM, $*P < 0.001$.

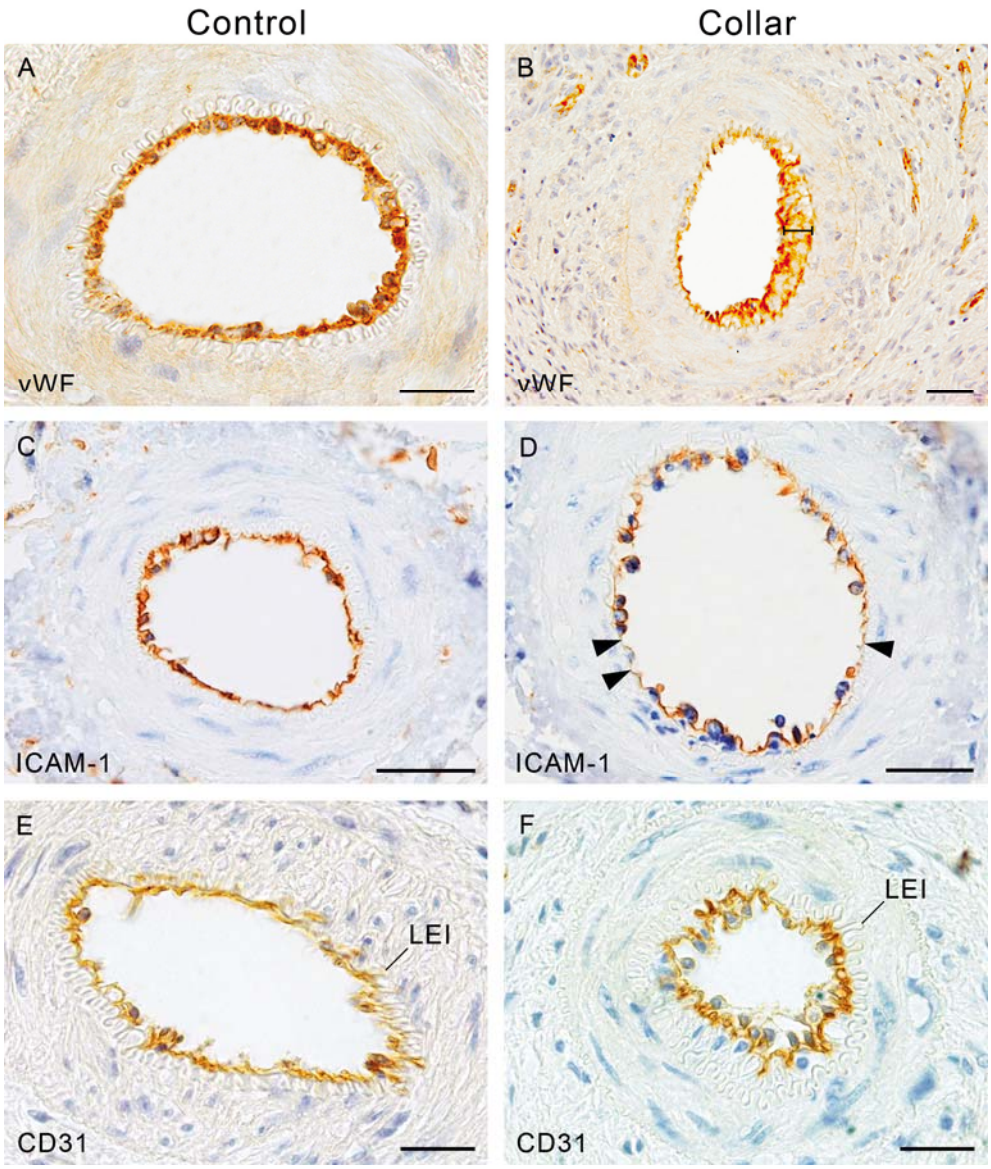

Figure 2. Endothelial cell markers in representative cross-sections from (A,C,E) sham-operated and (B,D,F) femoral arteries 7 days after collar placement that were stained with antibodies against (A-B) vWF, (C-D) ICAM-1 and (E-F) CD31. LEI indicates lamina elastica interna. Arrowheads show regions of endothelial cell detachment. Scale bars: A,E,F 20 µm, B,C,D 30 µm.

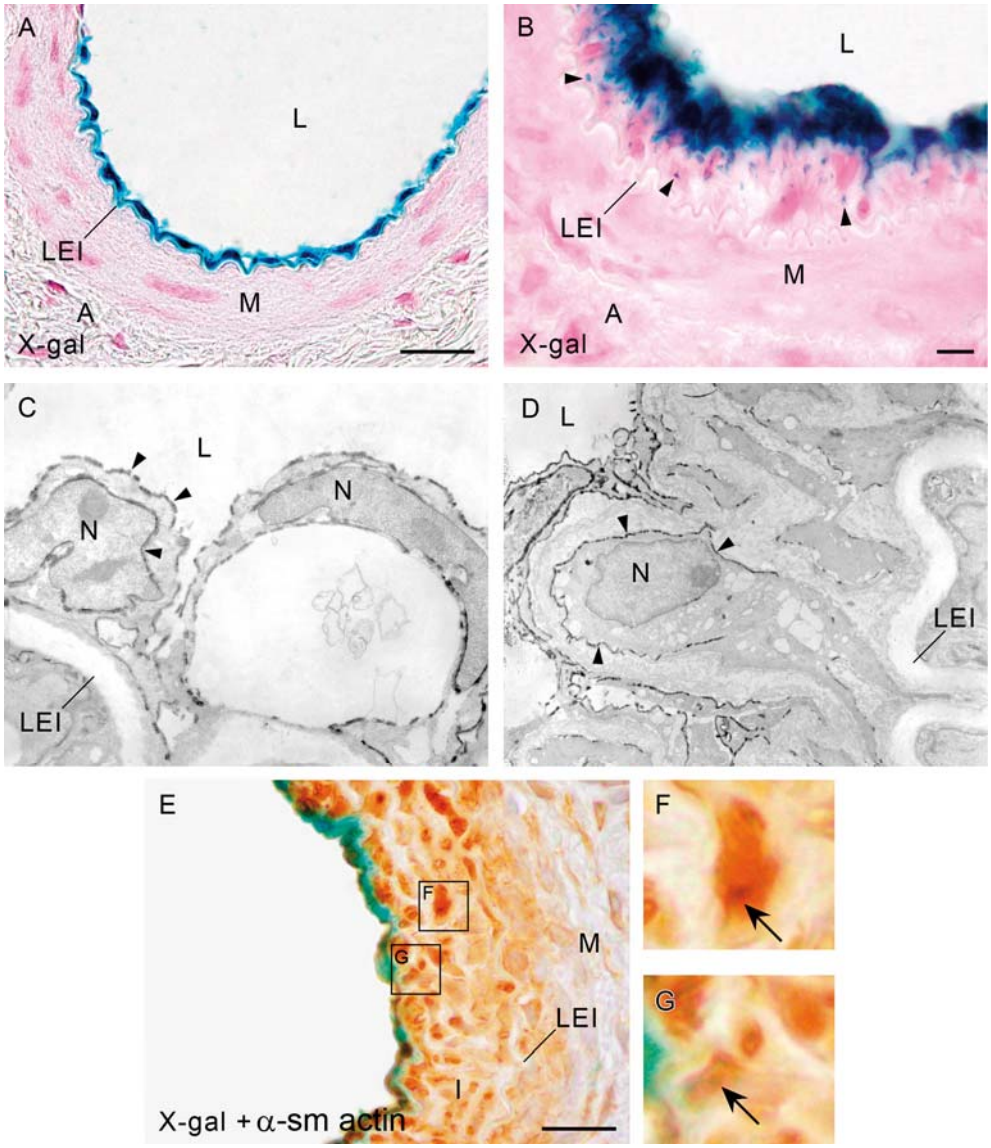

Figure 3. Beta-galactosidase expression in femoral arteries. Representative cross-sections from (A,C) sham-operated and (B,D,E) collared femoral arteries 2 weeks after collar placement. Beta-galactosidase expression in (A) endothelial cells and (B, small speckles, arrowheads) β-galactosidase expression in endothelial cells and mesenchymal cells. Transmission electron microscopic pictures of β-galactosidase in nuclear and cellular membranes of (C) endothelial cells and (D) cellular membranes of mesenchymal cells, magnification 5900x and 4400x respectively. (E) Beta-galactosidase and α-smooth muscle actin-positivity in mesenchymal cells. (F,G, α-sm actin) Detail of mesenchymal cells with α-sm actin and β-galactosidase within the same cell (arrow). L indicates lumen; LEI, lamina elastica interna; M, media; A, adventitia; N, nucleus. Scale bars: 20 µm.

Endothelial-to-Mesenchymal-Transformation

In Tie2LacZ reporter mice, β-galactosidase can be detected in tissues expressing Tie2, such as vascular endothelial cells (Figure 3A). Medial smooth muscle cells and adventitial fibroblasts of the vascular wall on the other hand, were completely devoid of staining. To precisely determine the localization of X-gal precipitate in the endothelial cells, expression was studied with transmission electron microscopy. In endothelial cells, the X-gal precipitate was found in the nuclear and cellular membranes (Figure 3C). Furthermore, staining was observed in the Golgi of these cells. There was no indication of phagocytosis as neither vacuoles nor a-specific depositions of β-galactosidase were found.

From 7 days after collar placement onwards, β-galactosidase was not only detected in the endothelial cells, but also in the neointima (Figure 3B). In these mesenchymal cells β-galactosidase expression was solely observed in the cellular membrane (Figure 3D). Staining of X-gal in the mesenchymal cells gradually diminished but could still be detected at 14 days after collar placement. A number of β-galactosidase-positive mesenchymal cells showed positivity for α-smooth muscle actin from 7 days onwards (Figure 3E-G).

The results indicate that endothelial cells have the capacity to undergo EMT. However, Tie2 is not exclusively expressed in vascular endothelial cells, but can also be detected in part of the bone marrow-derived cells. To exclude the contribution of bone marrow-derived cells to the neointimal lesions, we performed bone marrow transplantations from Tie2LacZ and ROSA26LacZ mice to wild-type recipients prior to collar placement. No β-galactosidase-positive cells were detected in the endothelial cell lining of the femoral arteries 14 days after collar placement in bone marrow recipients (Figure 4A). In addition, no β-galactosidase-positive cells were found within the neointima and media of the arteries. We only demonstrated X-gal positivity in the adventitia and perivascular space, the area between the adventitia and the position of the collar, of wild-type mice transplanted with bone marrow of Rosa26LacZ donors (Figure 4B).

Both macrophages and CD34-positive endothelial progenitor cells are derived from the bone marrow. The lack of X-gal positivity in the neointima and media suggested the absence of both cell-types in this region. Immunohistochemistry confirmed that macrophages (Figure 4C-D) and endothelial progenitor cells (Figure 4E-F) homed into the adventitia, but not towards the neointima and media of the collared femoral arteries.

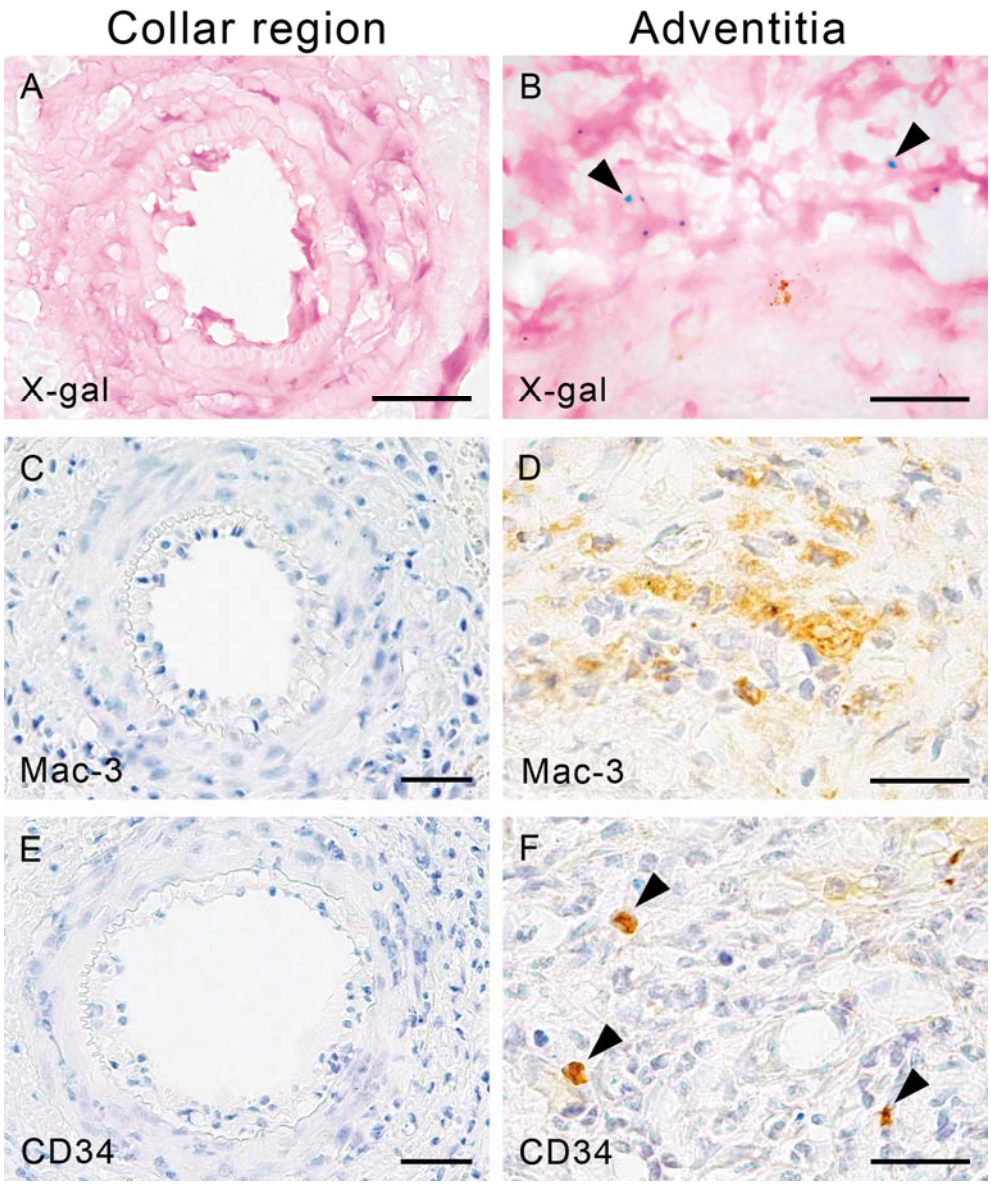

Figure 4. Bone marrow-derived cells in the adventitia. Representative cross-sections of the (A,C,E) intima and media and (B,D,F) adventitia 2 weeks after collar placement. (A-B) Beta-galactosidase expression, (C-D) presence of macrophages and (E-F) CD34-positive cells. Scale bars: 20 µm.

The Effects of Collar Placement on Tgf-β Signal Transduction

Since Tgf-β signaling is important in EMT, we examined the consequences of collar placement on Tgf-β signaling in the initiation phase of EMT, 3 days after collar placement. Intense nuclear PSmad2 staining was detected in endothelial and smooth muscle cells of sham-operated femoral arteries (Figure 5A). In contrast, PSmad2 staining was markedly decreased and even absent in numerous areas within collared femoral arteries (Figure 5B). Nuclear expression of PSmad1/5/8 was present in the endothelial and smooth muscle cells of sham-operated, as well as collared femoral arteries (Figure 5C-D).

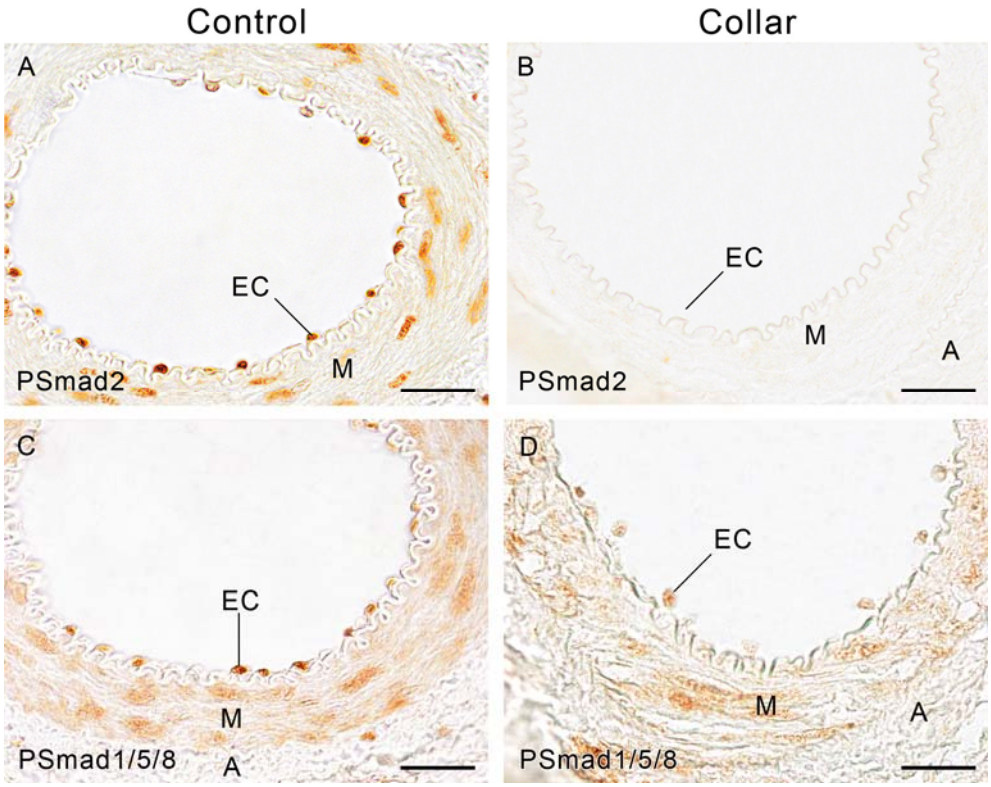

Figure 5. Tgf-β signaling pathway 3 days after collar placement. Representative cross-sections from (A,C) sham-operated and (B,D) collared femoral arteries were stained with antibodies against (A-B) PSmad2 and (C-D) PSmad1/5/8. EC indicates endothelial cell; M, media; A, adventitia. Scale bars: 20 μm.

Discussion

In the present study we show that endothelial cells in the vasculature of adult mice still have the capacity to undergo EMT. By doing so, endothelial cells contributed to the total pool of mesenchymal cells that composed the neointima. Recently, it has been reported that adaptive intimal thickening may be important in the development of early stage atherosclerosis as it was found that it preceded pathological plaque formation.[22] Furthermore, an important role has been ascribed to EMT in the process of cardiac fibrosis.[23] Constriction of the murine ascending aorta induced cardiac fibrosis and, in addition, evoked transdifferentiation of endothelial cells towards a fibroblast phenotype. Together, these data indicate that EMT could be important in development and progression of cardiovascular disease.

The early response to collar placement, at 3 days, was characterized by endothelial cell detachment and proliferation. Endothelial cell detachment is part of the process of cell scattering.[24] During cell scattering cell-cell adhesion complexes dissociate and the cells acquire cell motility. This process is a prerequisite for the occurrence of EMT. In addition to endothelial cell detachment, endothelial cell proliferation was observed. Smooth muscle cell proliferation was not detected. Our data are in accordance with a study that demonstrated that nonconstrictive collar placement solely induces intimal proliferation and has not adverse effects on the smooth muscle cell population of the media up to 2 weeks after collar placement.[9] We conclude that perivascular collar placement around the femoral artery is a suitable technique to study EMT.

Our data indicate that the initial population of mesenchymal cells is of endothelial origin. Besides positivity of the endothelial cells for β-galactosidase in the Tie2LacZ reporter mouse model, part of the bone marrow-derived cells can express Tie2. Bone marrow transplantations were performed to elucidate the role of circulating cells in our model and to exclude bone marrow origin of the mesenchymal cells. Bone marrow-derived cells were only detected in the adventitia. Our results are supported by the data of Xu and colleagues who in the same animal model showed that all bone marrow-derived cells were directed to the adventitia and none were found in the intima.[10] Thus, the β-galactosidase-positive mesenchymal cells are most likely of endothelial origin.

The β-galactosidase positivity seen in the mesenchymal cells was much less intense than that found in the endothelial cells. We showed that β-galactosidase staining was only present in the cellular membrane whereas in endothelial cells additional positivity was found in the nuclear membrane and Golgi. With subsequent cell divisions, the β-galactosidase that was present in the cellular

membranes diminished. These data indicate that Tie2 transcription in the mesenchymal cells is downregulated. Consequently, these cells lose endothelial cell-specific characteristics.

Mesenchymal cell characteristics on the other hand, were acquired. From 7 days after collar placement onwards a number of mesenchymal cells showed double positivity for β-galactosidase and α-smooth muscle actin. Our data are supported by a number of reports describing that during the initial phase of neointima formation the lesions contained α-smooth muscle actin positive cells.[8,9] Since transmission electron microscopy revealed that the internal elastic lamina remained intact up to 14 days, active contribution of medial smooth muscle cells to the neointima between 7 and 14 days after collar placement can be excluded.

LeClair and colleagues recently reported that normal mouse carotid arteries highly express the Tgf-β-related proteins PSmad1/5/8 and PSmad2/3 in the nuclei of the endothelial cells.[25] Our data in the femoral artery are in concordance with this observation. We found high levels of PSmad1/5/8, as well as PSmad2 in endothelial and smooth muscle cells. High expression of these Smad proteins appears to be related to a differentiated and quiescent state.[14] Tgf-β signaling pathways are not only associated with cell differentiation and quiescence, but also with the induction of EMT (reviewed by Zavadil[26]). Therefore, for in vitro experiments this cytokine has been generally used to initiate EMT.[2-4] Our data show that collar placement results in downregulation of Tgf-β/Alk5/PSmad2 signaling in endothelial cells. As a consequence, the balance between PSmad2/3 and PSmad1/5/8 shifted towards the Tgf-β/Alk1/Eng/Smad1/5/8 signaling pathway. Since the latter is associated with increased proliferation and migration of cells,[27] we postulate that Tgf-β signaling plays an important role in EMT in vivo. Additional studies on Smad proteins, Endoglin and the receptors Alk1 and Alk5 are foreseen to determine their role and the overall influence of Tgf-β.

In conclusion, the present study has provided evidence that adult endothelial cells in vivo have the capacity to undergo EMT. Furthermore, through EMT endothelial cell provide a first population of mesenchymal cells. We postulate that EMT may play an important role in the development and progression of cardiovascular disease.

Acknowledgements

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