In vivo transluminal microvascular endothelial cell seeding on balloon injured rabbit arteries

Cora HP Arts\textsuperscript{12}
Philip G de Groot\textsuperscript{2}
Nico Attevelt\textsuperscript{3}
Glenda J Heijn-Snyder\textsuperscript{2}
Hence JM Verhagen\textsuperscript{1}
Bert C Eikelboom\textsuperscript{1}
Jan D Blankensteijn\textsuperscript{1}

Department of Vascular- and Transplantation Surgery\textsuperscript{1}, Thrombosis and Haemostasis Laboratory, Department of Haematology\textsuperscript{2}, University Medical Center, Joint Animal Laboratory of the University of Utrecht\textsuperscript{3}, Utrecht, The Netherlands
Abstract

**Background:** Seeding venous endothelial cells (EC) onto damaged vascular surfaces attenuates the development of intimal hyperplasia. Unlike venous EC, fat derived microvascular endothelial cells (MVEC) do not require a culture step to increase the yield. In this *in vivo* study we investigated whether fat derived MVEC are suitable to reduce intimal hyperplasia and to increase patency after PTA.

**Methods:** Five rabbits were subjected to PTA of both iliac arteries. MVEC were isolated from the perirenal adipose tissue. One side was seeded transluminally with autologous MVEC, using a double balloon catheter. The contralateral side was sham seeded, and served as a control. Follow up was 4 weeks. Another rabbit was used for a feasibility experiment. This rabbit was subjected to a one-sided seeding procedure and was sacrificed after 1 week. In a seventh rabbit, a one sided PTA was transformed, and autologous labelled cells were injected in the distal aorta instead of seeded, follow up was 1 week. After sacrifice, vessels were removed for histologic investigation.

**Results:** The MVEC seeded artery of the pilot experiment was patent. All sham seeded arteries (5), with a follow-up of 4 weeks, except for one were patent. The patent ones showed moderate intimal hyperplasia. MVEC seeding (5), with a follow up of 4 weeks, resulted in two occlusions. In the patent MVEC seeded arteries intimal hyperplasia was present more extended than in the sham seeded arteries. Histology of the occluded vessels showed an organized thrombus. Both the patent MVEC- and sham seeded arteries were covered with an EC layer. Injected labelled MVEC were not found again on the deendothelialized artery.

**Conclusion:** In this study seeding of fat derived MVEC on damaged native arteries results in an increased development of intimal hyperplasia and a decreased patency. One of the reasons may be the presence of non-EC in the seeded cell population.
Introduction

Restenosis after percutaneous transluminal angioplasty (PTA) or endarterectomy is a common clinical problem. Intimal hyperplasia, responsible for the chronic aterial renarrowing, is a result of the migration and subsequent proliferation of vascular smooth muscle cells with associated deposition of extracellular matrix.\textsuperscript{1,2}

Endothelial cell (EC) seeding was originally introduced as a method to improve the patency of small diameter vascular prostheses.\textsuperscript{3} Since then, it has been demonstrated to decrease thrombogenicity and increase patency of vascular grafts.\textsuperscript{4,5}

EC seeding has also been proposed as a method to control the development of intimal hyperplasia after PTA and endarterectomy.\textsuperscript{6} Promising results were obtained in a small number of studies performed in rabbits or dogs. In these studies venous EC were seeded on deendothelialised carotid or ilio-femoral arteries.\textsuperscript{7-11}

A disadvantage of using veins is the limited yield of EC prohibiting immediate seeding after isolation.\textsuperscript{12} Cell culture is necessary in a special facility. This culture procedure is laborious and expensive, and cells cannot be used for an emergency intervention in case of an acute vessel occlusion.\textsuperscript{13,14}

Subcutaneous fat is a source of microvascular endothelial cells (MVEC).\textsuperscript{15} The yield is high enough and additional culturing is unnecessary. After seeding these cells on prosthetic grafts, in a dog model, thrombogenicity was reduced.\textsuperscript{16} A confluent EC layer was present on the grafts. A neointima still developed but less progressively.

Recent studies demonstrated that circulating EC (progenitors), from bone marrow, can contribute to angiogenesis and to covering of prosthetic grafts and deendothelialized surfaces.\textsuperscript{17,18}

The aim of this study was to investigate the effect of seeding fat-derived MVEC on damaged native arteries, and whether these cells can home to the damaged surface.
Methods

Animals
Rabbits, New Zealand Whites, weighing 3-3.5 kg, were used. They were given free access to water and standard rabbit chow, and were housed in an animal laboratory with alternating light and dark cycles. Presence of parasites or micro-organisms was checked every month, investigating blood, stool and coat of fur. Animal experiments were approved by the ethics committee of the University of Utrecht. The animal care complied with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. Washington: National Academy Press, 1996.

Design of the study
A seeding procedure of the common iliac arteries after angioplasty was performed using perirenal fat derived MVEC.
A One rabbit was used for a feasibility experiment: seeding of MVEC after unilateral PTA of the common iliac artery. The follow up was one week.
B Twelve rabbits were subjected to bilateral PTA of the common iliac arteries. One side was seeded transluminally with autologous MVEC, using a double balloon catheter. The contralateral side was sham seeded and served as a control. The follow up was 4 weeks.
C Three rabbit was subjected to a one-sided PTA. Autologous labelled MVEC were not applicated directly on the deendothelialized surface, but injected into the distal aorta, to investigate homing. The follow up was 1 week.

Anaesthesia
Identity number and weight were registered. Premedication, acetylpromazin and methadone, were given intramuscularly. Intravenous (i.v.) access was applied in the ear. I.v. anaesthesia, etomidate, was administrated by means of titration. Subsequently, intubation was performed and inhalation anaesthesia, O₂/NO₂ and halothane, were administrated. The animals were monitored by electrocardiography, intra-arterial blood pressure, capnography and body temperature. Before the incision an antibiotic (amoxicilline) were given i.v. and just before the PTA heparin was given i.v.
Postoperatively an analgesic, buprenorfine, was give, twice a day, on day 0, 1 and 2, and long acting amoxicilline was given on day 1 and 3.
**Isolation and culture of MVEC**

Blood, 10 ml was taken to obtain autologous serum. MVEC were isolated from the perirenal fat. Amounts of fat were incubated for 30 min at 37°C under continuous vigorous shaking with equal volumes of crude collagenase obtained from *Clostridium histolyticum* (Sigma-Aldrich Chemie, Steinheim, Germany) (0.4% w/v), BSA (Sigma) (0.4% w/v) in Phosphate Buffered Saline (PBS), pH 7.4. The digested fat was sieved and centrifuged (1200 rpm; 12 min; room temperature (RT)). The pellets were resuspended in Medium 199 (Gibco BRL, Life Technologies, Paisley, Scotland). The cell suspension was centrifuged again (1100 rpm; 5 min; RT) and the pellet was resuspended in sodding medium (Medium 199 with autologous serum in a ratio of 6:1).

The cells used for seeding were resuspended in such a way that the seeding density would be 5x10⁵/cm². We assumed the seeding surface to be 1.9 cm² (2 x π x radius x length). An exact cell count could not be made after the isolation procedure, since a counter was not available in the animal laboratory. Therefore, the yield of cells was estimated to be 10⁶/g of fat. A final count was made after finishing the seeding procedure.

The cells used for identification were seeded in a concentration of 10⁶ cells/cm² on fibronectin-coated glass coverslips. The cells used for genetic labelling were seeded in the same concentration on Retronectin® (Takara Biomedical Group, Shuzo Co, Shigo, Japan)-coated non-tissue-culture-treated plates.

MVEC were cultured in EBM-MV2 Bulletkit culture medium (Clonetics, BioWhittaker, Verviers, Belgium)/0.5 µM dibutyryl cAMP (Sigma).

**Identification of isolated cells**

Cells were incubated with Dil-Ac-LDL 10 µg/ml in culture medium for 4 hours. After several wash steps, cells were fixed with 3.5% paraformaldehyde (PFA). For immunohistochemical staining cells were fixed after confluence with 3.5% PFA/0.1% Tween. Rabbit anti-human von Willebrand Factor (vWF) (Dako, Glostrup, Denmark) was used and for visualization FITC-conjugated goat anti-rabbit antibody (Becton and Dickinson, Bedford, Mass., USA). Incubations were done for 45 min at 37°C, followed by three wash-steps of 10 min. Cells were visualized with confocal laser microscope (Leica TCS nt, Leica, Heidelberg, Germany).
Retroviral gene transduction of isolated cells

We used retroviral gene transduction, gene delivery via a replication deficient retrovirus, to tag cells with a marker gene which is readily detectable.\(^{19}\) Cells were transduced with truncated low affinity nerve growth factor receptor (LNGFR), a human receptor. Two days before start of the transduction, producer cells (Frapé-1, 1\times10^7 IU/ml) were plated in different concentrations.\(^{19}\) After 24h, producer cells with the highest concentration were covered with EBM-MV2 Bulletkit culture medium without hydrocortison. The next day this MVEC-medium was harvested, filtered, and after addition of cAMP and hydrocortison, applied to the MVEC. Transductions were repeated for three days. One week after isolation transduced and mock(non)-transduced cells were trypsinized, washed and resuspended for seeding as mentioned above. In total 3.8\times10^6 cells were injected.

Small amounts of cells were withheld to determined the transduction efficiency, defined as the percentage of cells that express the marker gene. Cells were stained with mouse anti-human LNGFR (200-3-G6-4 (20.4)) (American Type Culture Collection, Manassa, VA, USA)/ PerPE-conjugated rabbit anti-mouse IgG1 (Southern Biotechnology Association, Allebama, USA). Transduction efficiency was established with a flow cytometer (Calibur, Becton and Dickinson, San José, CA, USA).

Angioplasty and cell seeding

A piece of perirenal fat tissue was excised through a dorsolateral subcostal incision. After achieving haemostasis, the wound was closed with absorbable sutures (vicryl 4-0).

The right carotid artery was exposed, ligated distally, and a 5F sheath (Terumo Europe, Leuven, Belgium) was inserted through which a 3F curved catheter (Cordis Europe, Roden, The Netherlands) was introduced and positioned in the descending aorta, using contrast fluid, Telebrix (Laboratoire Querbet, Aulnay-sous-bois, France). The sheath was advanced over the catheter. To obtain an angiogram of the distal aorta and iliac arteries, the curved catheter was positioned into the distal aorta and contrast fluid was injected by hand. Angiograms were made using a C-arm (Philips Medical Systems, Best, The Netherlands) and the vascular tree was inspected to find a suitable spot for an artery without side-branches for at least the length of the segment excluded from the circulation when the balloons were inflated (2 cm). Position was documented using a radiopaque ruler. Subsequently the curved
catheter was exchanged for a 3 mm balloon angioplasty catheter (Savvy, Cordis; balloon length 2 cm). The angioplasty catheter was introduced over a guide wire. We started at random with PTA of the right or left iliac artery, depending on the artery in which the balloon angioplasty catheter was advanced first. Dilatation was performed by inflating the balloons to 8 atmospheres pressure for 30 seconds. Post-angioplasty angiograms were made, and the seeding catheter was introduced.

The seeding procedure was performed transluminally using a double balloon catheter as described by Nabel et al.20 A 4F double balloon catheter (Hutson dual balloon catheter, H-4F.DB, Krijnen Medical, Beesd, The Netherlands) was modified by R. Mansfeld Beck, University Medical Center Utrecht, The Netherlands. This final catheter existed of two compartments. Through one compartment, two low pressure latex balloons could be filled. The distance between both balloons, when filled, was 2 cm, the exact length of the damaged artery after PTA. Through the other compartment, the medium with cells or without cells (sham seeding) could be introduced between both filled balloons. We started at random with EC seeding or sham seeding, depending on whether the isolation procedure of cells had finished already.

The balloons of the seeding catheter were filled with contrast and inflation was performed to 2 atm. A volume of 0.2 ml was introduced into the isolated arterial segment, followed by 0.1 ml every 5 min in case of lost resistance to replenish lost by leakage. The incubation was performed for 30 min with the animal in various positions to obtain circular distribution of EC. After this the blood flow was restored by deflating the balloons and removing the catheter. PTA and seeding of the contralateral side was performed using the same procedure.

In case of the ‘homing experiment’ cells were injected in the distal aorta. At the end the sheet was removed and the proximal carotid artery ligated. The skin was closed using absorbable sutures.

Rabbits were heparinized during catheterisation (100 IU/kg), no anticoagulants were give postoperatively.

**Sacrifice**

The rabbits were premedicated and i.v. access was obtained. Heparin was administrated iv. Termination was performed using sodium pentobarbital. A midline abdominal incision was made and the distal aorta and iliac arteries were dissected. One canula was introduced into the distal aorta and the proxi-
mal aorta was ligated. Another canule was introduced into the caval vein and the proximal part was ligated also. The distal arterial tree was first flushed with PBS for 10 min, followed by in situ pressure perfusion with 2% paraformaldehyde at 80 mmHg for 20 min. The terminal aorta and iliac arteries were excised and placed in 4% formaldehyde for at least 24 hours.

**Histopathology and immunohistochemistry**

Paraffin slides were made. For every vessel, 6-8 cross sections were taken over a the length of 2 cm where PTA and seeding were performed. Haematoxylin and eosin (HE) and immunohistochemical stains were performed.

For each rabbit the intimal thickness was determined as follows: For every cross section (6-8 per vessel), at a regular distance of 300 µm, the thickness was measured. Measurements were performed with a light microscope equipped with a JAC-CCD camera (Copenhagen, Denmark) coupled to a matrox frame grabber (Matrox Electronic systems Ltd., Quebec, Canada) using Optimas 6.0 software (DVS, Breda, The Netherlands). Immunohistochemical stains were preceded by endogenous peroxidase blocking and citrate boiling, or Triton-serum blocking as in the case of LNGFR-staining. Endogenous peroxidase blocking was performed with 1.5% H₂O₂ in phosphate-citrate buffer (citric acid mono-hydrate 8.32 g/l, Na₂HPO₄ 21.52 g/l; pH 5.8±0.1) for 15 min. Citrate boiling was done in citrate buffer (Na₃citrate dihydrate 2.94 g/l; pH 6.0) for 10 min. Triton-serum blocking was done with 0.4% Triton X-100/ 5% horse serum in PBS for 1 hour. As primary antibodies mouse anti-human CD31 (DAKO), mouse anti-human α-actin (DAKO) and mouse anti-human LNGFR were used. Peroxidase/DAB stainings were done with Vectastain Elite ABC Kit and DAB kit for peroxidase staining (Vector Laboratories, Burlingame, CA, USA), according to the manufactures’ recommendations.

**Statistical analysis**

Results are presented as the median (quartile range).
Results

Isolation and identification of MVEC

From the perirenal fat 8 (7.4-10) grams of fat was obtained (n=16). The yield of MVEC was 9.1 (7.2-11.5) x10^6 cells/g (n=16). When a part of the cells were cultured, no growth failures were observed.

The percentage of EC was estimated to be between 30 and 40% based on fluorescence for vWF, and uptake of Ace Dil. In fat derived cell cultures MVEC were situated in groups (fig. 1). The other cells had a fibroblast-like morphology, elongation with branched shoots without contact inhibition.

The seeding density was 6.4 (4.6-9.2) x10^5/cm^2 (table 1).

Operative outcome

Nine rabbits of the 16 died during the procedure: blow out rupture of the stomach (n=1), dissection of the aorta (n=2), dissection of one of the iliac vessels (n=3), technical failure of the ventilator (n=1), aorta spasm (n=1), and rupture of the carotid artery during removal of the sheet (n=1). Seven of the rabbits that had died were intended for an unilateral MVEC seeding procedure and a contralateral sham seeding procedure, and two rabbits were intended for an injection procedure with labelled cells.

Figure 1
Immunocytochemistry of a confluent layer of first passage rabbit MVEC. Cells were identified as EC by:
A. Uptake of Dil-Ac-LDL (magnification, x195)
B. Positivity for the EC-specific marker against vWF (magnification, x 305)
Dissection of the aorto-iliac tract was related to the seeding catheter twice. One time, one of the balloons emptied into the other. The other time, adhesion of the double balloon catheter to the vessel wall occurred.

In the other seven rabbits (including one feasibility experiment) the procedure was uncomplicated, leaving five rabbits for the ‘seeding procedure’ and one for the ‘homing procedure’ (fig. 2).

**Patency of the MVEC and sham seeded arteries**

The MVEC seeded native artery of the rabbit from the preliminary study with a follow up of 1 week was patent. Of the five rabbits present for analyses of MVEC seeding, with a follow up of 4 weeks, one rabbit had a short-term paresis of the left lower paw. In three rabbits both the MVEC- and the sham seeded native arteries were patent. In one rabbit the sham seeded artery was patent, but the MVEC seeded artery occluded. In another rabbit, both the MVEC- but also the sham seeded arteries was occluded. All occlusions occurred exactly at the side of the PTA. Both occlusions of the EC seeded artery occurred in the rabbits with the highest EC seeding densities (table 1).

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**Figure 2**

Fluoroscopy:
A. Normal aorto-iliacal tree
B. PTA process
C. Seeding process, using a double balloon seeding catheter
In vivo seeding of denuded arteries

The deendothelialized iliac artery of the rabbit of the ‘homing experiment’ remained patent after one week of follow up.

**Histopathology**

In case both the MVEC seeded and the sham seeded vessels were patent (n=3), the median intimal thicknesses were compared. For the sham seeded arteries the median intimal thicknesses were 60, 61, and 59 µm. For the EC seeded arteries the median intimal thicknesses were 123, 105, and 92 µm, respectively (table 1).

**Immunohistochemistry**

All patent seeded and sham seeded native arteries were covered with an almost confluent CD31-positive EC layer. Cells present in the neointima were α-actin-positive, classifying them as myofibroblasts (fig. 3).

The occluded native arteries were filled with SMC, myo-fibroblasts, connective tissue, and neovascularization.

**Homing of MVEC to damaged native surfaces**

The transduction efficiency of MVEC with LNGFR was 93% (fig. 4). After one week, in none of the 6 cross-sections over the length of 2 cm, where deendothelialization of the common iliac artery had been performed, labelled cells were found.
Immunohistochemistry of a patent MVEC and sham seeded deendothelialized artery of the same rabbit with 4 weeks follow up (magnification, x85):

A. Anti-CD31 staining of the MVEC seeded artery: Most of the cells present in the luminal monolayer are positive (brown) and so identified as EC.

B. Anti-α-actin staining of the same artery: The cells present in the intima are positive (brown) and so classified as myofibroblasts.

C. Anti-CD31 staining of the sham seeded artery: Most of the cells present in the luminal monolayer are positive (brown) and so identified as EC.

D. Anti-α-actin staining of the same artery: The cells present in the intima are positive (brown) and so classified as myofibroblasts.
Discussion

EC seeding, introduced to reduce thrombogenicity of vascular grafts, has also been proposed to reduce intimal hyperplasia and thus to increase patency of deendothelialized surfaces. Promising results were obtained with venous EC in animal models. In this study we have investigated, in a rabbit model, whether fat derived MVEC are suitable as an alternative to reduce intimal hyperplasia and increase patency of deendothelialized surfaces. We found that MVEC, isolated from the perirenal fat, consisted of a combination of EC and fibroblast-like cells. Of the five MVEC seeded arteries two were occluded, and of the five sham seeded arteries one. In all patent MVEC seeded arteries intimal hyperplasia was present more pronounced than in the patent sham seeded arteries. After injection of labelled EC in another rabbit, to investigate homing, injected MVEC were not found on the deendothelialized surface. Nine rabbits intended for treatment as well did not survive the procedure. Only in two of the nine cases dead was directly related to the seeding procedure.

Histology of the occluded arteries showed that the occlusion existed almost completely of myofibroblasts, SMC, connective tissue, and neovascularization. No signs were observed in the rabbits when an iliac artery was occluded. So it was impossible to conclude whether early thrombus formation had occurred, which subsequently organized, or late occlusion had taken place due to accelerated development of intimal hyperplasia. The occlusions and accelerated formation of intimal hyperplasia of MVEC seeded native arteries seems to be related to the seeding procedure. In this rabbit model, it appears that the procoagulant and growth stimulating func-
tions of the seeded cells overpowered the expected anticoagulant and growth inhibiting functions. In a dog study we showed seeding of MVEC on prosthetic grafts is successful.\textsuperscript{16} The thrombogenicity decreased and surfaces were covered with a smooth layer of EC. On the other hand, we also showed that contaminating cells were present in the transplant, and that these cells contributed to development of intimal hyperplasia. In the isolates of rabbit MVEC contaminants were present as well. Seeding of pure venous EC on deendothelialized surfaces reduced intimal formation and increased patency.\textsuperscript{7,10} We postulate therefore that the presence of contaminating cells in the EC-isolates contributed to the increase of intimal hyperplasia after seeding of deendothelialized surfaces.

Not only the presence of contaminating cells in the population of seeded cells but also the number of seeded MVEC seems to influence the development of intimal hyperplasia. In most studies using venous EC, a seeding density of $2 \times 10^5$ was used.\textsuperscript{21,22} We planned to use a density around $5 \times 10^5$, as a previous study has shown that a confluent layer could be formed in 30 min, when at least $5 \times 10^5$ cell/cm$^2$ were seeded.\textsuperscript{23} In addition, not all of our cells were EC, so we decided to use a higher seeding density to be sure that EC were still seeded at a density high enough to reach confluency. In some rabbits the amount of seeded cells was larger than $5 \times 10^5$/cm$^2$, since we could only count the cells after the seeding procedure. Occlusions occurred related to the higher seeding densities.

Both the MVEC seeded as well as the sham seeded arteries were covered with an almost confluent EC layer. This means that on the sham seeded arteries spontaneous reendothelialization occurred, as was demonstrated earlier by Conte et al, who found 70% recovery of EC covering.\textsuperscript{21} Animals are known to reendothelialize easily.\textsuperscript{24} EC originate from the adjacent intact native artery, from the circulation, or from the tissue surrounding the vessel.

In case of seeding prosthetic grafts a ‘pressure sodding technique’ is used.\textsuperscript{15} Cells are pressed into the crevices of the graft. A deendothelialized artery is not porous so that the pressure sodding technique cannot be used. The most obvious way to perform attachment of the cells to the vessel is transluminal seeding of a temporary closed vessel segment using gravity. An alternative is based on recent publications about circulating progenitor EC, originating from bone marrow. Studies demonstrated that these cells can contribute to angiogenesis and covering of prosthetic grafts and deendothelialized surfaces.\textsuperscript{17,18} We could not demonstrate the homing of injected MVEC to the damaged arte-
rial surface. However, only one rabbit was available and a follow up of one week might have been too short. Moreover, an immunogenic marker was used and cells might have been removed by the immune system. In addition, only EC progenitors might be capable of homing and not mature EC. Finally, the presence of labelled cells might have been missed, since only one slide was made every 0.33 cm.

From the present study we conclude that seeding deendothelialized arteries after PTA with fat derived MVEC results in an increase of intimal hyperplasia and in a reduced patency. We could not demonstrate the occurrence of homing of mature MVEC. Contaminants from the transplant likely contribute to intimal hyperplasia associated with fat derived MVEC seeding. The procedure should be repeated with purified fat-derived MVEC.

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