Evaluating intimal hyperplasia under clinical conditions

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Abstract

OBJECTIVES: Open arterial revascularization using venous segments is frequently associated with the development of intimal hyperplasia (IH), leading to severe restenosis and graft failure. The lack of treatment to prevent this pathology is a major problem. Therefore, we generated a new porcine model, which closely mimics the clinical development of human IH, to test the therapeutic potential of candidate drugs.

METHODS: A patch of jugular vein was sutured to the right common carotid artery of pigs, to expose the vein to haemodynamic conditions of the arterial bed. Four weeks after surgery, the operated vessels which received no further treatment (the control group) were compared with (i) contralateral, non-operated vessels (the healthy group); (ii) vessels of pigs that received a perivascular application of a drug-free microparticle gel (the placebo group) and (iii) vessels of pigs that perioperatively received the same gel loaded with 10-mg atorvastatin (the atorvastatin group).

RESULTS: When compared with non-operated vessels, all operated segments displayed a sizable IH which was thicker in the venous patch than in the host artery. These alterations were associated with a thickening of the intima layer of both vessels in the absence of inflammation. The intima/media ratio has been significantly increased by 2000-fold in the vein patches. Perivascular application of atorvastatin did not prevent IH formation. However, the drug increased the adventitial neovascularization in the operated vessels.

CONCLUSIONS: The novel porcine model allows for monitoring IH formation under haemodynamic conditions which mimic clinical situations. It should facilitate the screening of innovative treatments to prevent restenosis.

Keywords: Intimal hyperplasia • Pig • Jugular vein • Carotid artery • Smooth muscle cells • Proliferation • Atorvastatin • Perivascular administration

INTRODUCTION

More than 1 million vascular grafts are implanted annually around the world. For peripheral bypass, saphenous vein graft is the conduit of choice [1]. Up to 50% of these grafts fail within the 1st 18 months following surgery due to the development of intimal hyperplasia (IH) at the anastomosis site [2]. This alteration, which results from the exposure of the vein to the arterial haemodynamic environment, is characterized by the migration of proliferative smooth muscle cells (SMCs) into the intima layer, leading to extracellular matrix deposition which thickens the vascular wall and often results in graft occlusion [3].

We have previously shown that the sustained, perivascular release of atorvastatin (ATV) efficiently inhibits the development of IH in a mouse carotid model [4], raising the hope that an analogous intervention may become a clinically relevant therapeutic strategy. However, the rodent model [5–7] does not closely mimic haemodynamic conditions observed in humans [3, 8], calling for an alternative model in larger animals [9, 10].

The use of the venous patch to enlarge stenotic arteries is often performed in vascular surgery, for example, in carotid bifurcations [10]. In our model, the main advantage of such a venous patch is to provide the unique possibility to compare venous and arterial walls, at the same vascular site. The main feature of the new model is to use a venous patch plasty of an artery.

As there is no diameter mismatch (which is usual with venous segment interposition), the venous and arterial walls can be compared at the very same vascular site, i.e. under strictly identical flow and pressure conditions. Therefore, we developed a novel porcine model in which IH is induced in a vein patch sutured to an artery. The development of IH in this model was assessed after 1 month, period matching the development time of IH as...
reported in humans [3], in the absence and presence of perivascular applied ATV.

METHODS

In vivo model

Adult Yorkshire domestic pigs of body weight approximately 50 kg were used in compliance with the Swiss Federal Law on the Protection of the Animals and according to a protocol authorized (No. VD2870a) by the veterinary authority of the Canton of Vaud. The animals were anesthetized by an intramuscular injection of 10 mg/kg ketamine, 0.1 mg/kg xylazine and 2 mg/kg atropine. Induction was done by 3.5–5 mg/kg propofol and 1.5 mg/kg portalgesic. General anaesthesia was maintained using 1–2% isoflurane via a tracheal intubation.

After a longitudinal skin incision of the right neck side, the subcutaneous and muscular tissues were dissected to expose the common carotid artery and the internal jugular vein (Fig. 1A). A 3-cm-long internal jugular vein segment with a 0.5–1.0-cm diameter was harvested between a proximal and distal ligation of the vessel. The internal jugular vein was left occluded. This segment was flushed with saline solution and opened longitudinally to obtain the venous patch. Following an intramuscular injection of 50 Ui/kg heparin, the common carotid was clamped proximally and distally using vascular clamps and subjected to a 3-cm-long longitudinal arteriotomy (Fig. 1B). The venous patch was sutured to the arterial wall using 6.0 polypropylene continuous stitches (Fig. 1C), orienting the endothelium towards the lumen of the artery. The arterial blood flow was then restored by declamping the common carotid artery. After declamping the common carotid and internal carotids, we assessed the quality of the blood flow by feeling the pulsation at the internal and common femoral arteries, as it is routinely made in clinical settings. The presence of pulsations in all animals indicated that the vascular surgery did not markedly alter the arterial flow. Moreover, an arteriography was performed at the end of the procedure to confirm the restoration of such a flow. Whenever a blood leakage was observed after declamping of the common carotid, the suture line was sealed with additional non-resorbable stitches.

The operated pigs were randomly attributed to 3 groups, each comprising 8 animals: in the control group, no drug was applied; in the placebo group, a drug-free hydrogel-microparticle formulation was perioperatively applied on the entire grafted surface, covering both the venous patch and suture lines (Fig. 1D); in the ATV group, the same formulation supplemented with 10-mg ATV was applied. The details regarding the preparation of the formulations is given in the Supplementary Material, supplementary methods. The muscular and subcutaneous layers of the neck were closed using uninterrupted sealing stitches to create a confined space around the venous patch, aimed at limiting the spreading of the gel. The wound was dressed using the Opsite® spray. Postoperative analgesia was provided by 1 intramuscular injection of 0.01 mg/kg buprenorphine hydrochloride and a patch application of 50-μg fentanyl. Animals were then

Figure 1: Representation of the novel porcine model. A schematic representation (upper panels) and corresponding photographs of the porcine model (lower panels). (A) The right jugular vein and carotid artery (separated by blue surgical loops) were exposed. (B) A longitudinal incision was performed on the artery, and a segment of vein (b) was longitudinally cut. (C) The vein was sutured to the longitudinal incision of the artery. (D) A microparticle-containing gel (white) was applied on the vein patch of the placebo (a drug-free formulation) and the atorvastatin groups of pigs (formulation containing 10-mg atorvastatin).
Figure 2: Intimal hyperplasia consistently developed in all grafted venous patches. Upper panel: representative histological sections stained for elastin (Miller Elastic van Gieson stain) of carotid arteries without (healthy) and with a venous patch (the operated, placebo and ATV-treated groups). Intimal hyperplasia consistently developed in all the grafted venous patches. The areas boxed by the white rectangles are shown at higher magnification in the panels of the 2nd and 3rd rows. Double-headed arrows show the thickness of intima and media layers. In the right column, healthy non-operated contralateral arteries and veins illustrate normal organization of the vessel wall. Lower panel: morphometric measurements of intima and media thickness of the carotid host arteries (left panels) and the venous patches (right panels) revealed no significant difference between the ATV-treated, control and placebo pigs. Bars show the mean ± SEM values of 6–8 samples. Values were compared using 1-way analysis of variance (ANOVA) with Tukey’s multiple comparisons: **P < 0.01, ***P < 0.001, ****P < 0.0001 when compared with the healthy (non-operated), contralateral vessels (solid black columns). A: host artery; ATV: atorvastatin; i: intima; m: media; V: venous patch.
Figure 3: The cellular architecture of the intima layer of venous patches and host carotid arteries was not altered by the ATV treatment. (A) Immunostaining with an antibody against alpha-smooth muscle actin (SMA) is presented. Surgery did not alter the density of SMCs in the carotid media. A similar SMC density was found in the enlarged intima of the vein grafts. The ATV treatment did not modify this parameter, as judged by morphometry (right panels). The areas boxed by the black rectangles are shown at higher magnification in the panels of the 2nd and 3rd rows. (B) Double immunostaining for desmin (brown) and vimentin (purple) showed the presence of vimentin-positive fibroblasts and of vimentin- and desmin-positive myofibroblasts in the hyperplastic intima of all venous patches. The areas boxed by the black rectangles are shown at higher magnification in the panels of the 2nd and 3rd rows. The density of these cells was not modified by the ATV treatment (right panels). (C) The density of contractile SMCs in the carotid media was reduced after surgery, as judged by an immunostaining of smoothelin. These cells were also present in the hyperplastic intima of the venous grafts, at the same density observed in the carotids. The areas boxed by the black rectangles are shown at higher magnification in the panels of the 2nd and 3rd rows. This density was not affected by the ATV treatment (right panels). Data are presented as the mean values of 6–7 animals. Each bar represents mean ± SEM. Differences between groups were tested by 1-way analysis of variance (ANOVA) with Tukey’s multiple comparisons: *P < 0.05 and ***P < 0.001, when compared with the healthy vessels. A: arterial host; ATV: atorvastatin; V: venous patch.
returned to the farm, where they received 1750-mg amoxicillin (antibiotic) daily associated with 250-mg clavulanic acid (antibiotic useful for the treatment of a number of bacterial infections) and 200-mg Aspegic (lysine acetylsalicylate, an antiplatelet drug) for 5 days after surgery. The animals were fed a standard diet, and their temperature and weight were monitored daily. After 28 days, the animals were sacrificed with a lethal intravenous injection of barbiturate, and samples of left and right common carotids, jugular veins, thymus, sternocleidomastoid muscle, neck skin, biceps femoris muscle, heart, lungs and liver were collected to assess whether the drug induced tissue alterations and either fixed in 10% buffered formalin (vessels) or quickly frozen (all other organs). No residual gel was observed around the venous patch at the end of the 28-day-long experiment.

Immunohistochemistry

After deparaffinization and heat-mediated antigen retrieval, representative sections of healthy and operated carotids were incubated for the detection of (i) alpha-smooth muscle actin (SMA), using the Dako A0851 (diluted 1:300), a DAB Map detection kit and a Ventana amplification kit; (ii) desmin, using the Dako M0760 (1:40), a DAB Map detection kit and a Ventana amplification kit; (iii) vimentin, using the Dako M0725 (1:40) and a RedMap (Ventana) detection kit; (iv) ETS-related gene (ERG), using the Abcam ab133264 (1:400), a DAB Map detection kit and a Ventana amplification kit. ERG was the selected immunohistochemical marker due to its nuclear reactivity, which provides less non-specific staining than cytoplasmic markers such as CD31 and CD34. Moreover, as macrophages also express CD31, and as
some mesenchymal cells express CD34, ERG provides a highly specific staining of endothelial cells; (v) smoothelin stain, using the Santa Cruz Sc-23883 (1:100), the Abcam ab133469 (1:500) and a Ventana Rb-OmniM kit. All slides were counterstained using haematoxylin and bluing reagents (Ventana). Two controls, in which we omitted either the primary or the secondary antibody, were included in each experiment.

For quantitative analysis, 1 section immunostained for one of the antibodies listed above was used from each animal.Slides were digitized using an Axio Scan.Z1 slide scanner (Zeiss, Germany). Snapshots were extracted using the ZEN 2 software and analysed using the Definiens 2.4.2 bright field software (Tissue Studio, Germany). The media and intima layers of both arteries and veins were defined manually, and the software was set to score the number of cells positively stained by each antibody. For the analysis of vasa vasorum, microvessels were manually scored, at a 10× magnification, within the adventitia layer of SMA-stained vessels, using the Leica Qwin® software (Leica, Switzerland).

**Statistical analysis**

Statistical analysis was performed using the GraphPad Prism 7.02 software. Outliers were removed by the 'Robust regression and
Outlier removal method with $Q = 1\%$ [11]. Data are represented as mean ± SEM. Morphometry data were compared using 1-way analysis of variance (ANOVA) with Tukey’s multiple comparison tests: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (***) when compared with the healthy (non-operated) contralateral vessels.

RESULTS

Thrombosis was observed macroscopically at the time of sampling in the venous patch and in the common carotid artery on 2 control and 2 ATV-treated animals which were, therefore, excluded. In all animals, at 4 weeks, no residual gel was observed. Analysis of the other 20 animals showed a significant increase ($1.4 ± 0.19$ mm, $P < 0.0001$) development of IH on the venous patch but not on the host artery of all operated animals (Fig. 2), when compared with controls. The media thickness also increased by 33% ($P < 0.0001$) in the vein patches but not in the host carotids, when compared with that evaluated in the contralateral, non-operated healthy vessels. As a result, the intima/media ratio markedly increased by 2000-fold ($P < 0.0001$) in the vein patches but not in the host carotids. The development of IH in the vein patch was similar in the control, the placebo and the ATV groups.

Immunostaining revealed an SMC-rich neointimal area in the venous patch of all operated groups (Fig. 3A). In the host artery of the control group, we further observed a trend for decrease in contractile cells, whereas secretory fibroblasts increased by 79%, $P < 0.05$ and myofibroblasts showed a tendency for increase (Fig. 3B). The changes observed for contractile cells were confirmed by smoothelin immunostaining (Fig. 3C). None of these cellular patterns was significantly altered by the presence of ATV. However, the presence of the drug showed a tendency for increase of the density of vasa vasorum within the adventitia layer of venous patches, when compared with the lower levels observed in healthy and operated carotids and untreated venous patches (Fig. 4). Immunostaining for ERG revealed a continuous line of endothelial cells along the lumen of all venous patches and host arteries (Fig. 5). Morphometry showed that the numerical density of these cells was not significantly modified by the exposure to ATV (Fig. 5).

Four weeks after surgery, livers and sternocleidomastoid muscles of the control, placebo and ATV groups showed a normal architecture and did not feature signs of inflammation in either placebo or ATV-treated animals (Fig. 6). The 2 organs also retained minimal and comparable levels of ATV (Fig. 6), in spite of their different distance from the surgery site. Histology also
showed no sign of inflammation in the hyperplastic intima of the veins and the host carotids of all animals, whether treated with ATV or not and demonstrate that the overall structure of all the organs studied was quite alike that of controls (Fig. 7). However, few lymphocytes were consistently observed in the adventitia layer of these vessels (Fig. 7).

**DISCUSSION**

Pigs have a cardiovascular anatomy and physiology similar to those of humans, with whom they also share genetic similarities [12]. Therefore, they provide a useful model to longitudinally monitor experimental cardiovascular pathologies, mimicking those observed in humans [12, 13]. Herein, we generated a porcine model of venous patches exposed to arterial haemodynamic conditions to explore the development of IH and to test new therapeutic approaches against this alteration.

The model is innovative, given that pig lesions have so far been reported to be more ‘thrombotic’ and less ‘proliferative’ than those observed during the restenosis of human vessels [14]. Specifically, the new model allows for a simultaneous evaluation of IH in both the host artery (here a carotid) and the venous graft (here a jugular vein) at a very same site, i.e. under conditions in
which both the artery and vein are submitted to identical haemodynamic conditions, including potential changes in blood flow and turbulence which may result from the surgery. Furthermore, the model preserves the non-operated vessels of the contralateral side of the neck, providing an essential internal control, and features a surgical success rate much higher than previously reported for vein to artery interpositions [15]. Eventually, the model closely mimics clinical conditions as venous patches are frequently used in arterial surgery and carotids are a common site for such a corrective surgery [10]. Even though patches from saphenous veins are usual in the human clinic, we chose here to use jugular vein segments, given that saphenous veins of pigs are rather small in diameter, a parameter which, per se, increases the risk of thrombosis after angioplasty.

Using this model and when compared with the contralateral control vessels of the very same animals, we consistently observed the development of IH and a thickening of the media layer, in both the operated arteries and veins. However, IH was significantly more severe on the venous graft than on the host artery, in keeping with the larger remodelling of the venous wall, which is induced by the exposure to haemodynamic conditions of the arterial bed [16, 17]. Specifically, the ratio of intima to media, a parameter which has been associated with an increased risk of atherosclerosis and carotid stenosis [18], was more increased in the venous patch than in the host artery due to increases in the number of SMCs, myofibroblasts, and secretory fibroblasts. These findings are in agreement with the data we reported in previous publications, aimed at defining the cellular mechanisms leading to IH, including in an ex vivo system in which segments of human saphenous veins were exposed to arterial conditions [16, 17, 19, 20]. The new data document that, over a relatively short time period (28 days), the new pig model allows for the longitudinal monitoring of IH development under haemodynamic conditions which closely mimic those prevailing in the vessels submitted to angioplasty in the human clinic and which faithfully reproduce the cellular landmarks of the pathological alterations of human veins grafted on the arterial compartment.

We previously reported that a perivascular, sustained release of ATV could efficiently inhibit IH [5] in a mouse carotid ligation model [6, 7], a model which, however, does not mimic the open surgical revascularization and haemodynamic conditions of arteriovenous bypass on humans. We, therefore, tested whether ATV could also favourably decrease the development of IH in our innovative porcine model. To this end, we perioperatively applied around the operated vessels a formulation of ATV-loaded hydrogel, which, in vitro, sustained a continuous release of the drug for over 20 days (Supplementary Material, Figure S 1). The development of IH in the animals receiving the ATV-containing formulation was comparable to that observed in the placebo or control groups. Nevertheless, the pigs that received the ATV-treatment featured an increased density of vasa vasorum within the adventitia layer of the venous patch, a change which was not observed in the animals of either the control or the placebo groups. The unaffected IH could be due to the modest dose of ATV we tested in pigs, which was chosen based on previous static studies [21–24] and which was significantly lower than that shown to be effective in rodents [5]. Still, the increase in vasa vasorum indicates that this dose was effective in those tissues that were close to the site of drug application. These findings are consistent with the positive effect of statins on the proliferation and differentiation of endothelial progenitor cells, which are required for neoangiogenesis [25, 26]. Our histological analysis did not demonstrate important inflammatory signs within the operated blood vessels and within the organs adjacent or distal from the site of the perivascular ATV application, which, again, is consistent with the broad anti-inflammatory effects of statins [27, 28]. If the oral administration of these drugs may be complicated by organ toxicity [29], such alterations were not detected in our study. On this ground, and given that the effect of orally administered statins on angiogenesis is dose-dependent [30], future studies could safely investigate whether larger doses of ATV formulations could inhibit IH. Finally, the release of effective drugs should be sustained for at least few weeks immediately following the angioplastic surgery, which our previous [5] and current data suggest is achievable with a perioperative, perivascular application.

CONCLUSIONS

Our data show that the new porcine model we developed allows for monitoring the development of IH under haemodynamic and vein adaptation conditions which closely mimic the situations prevailing in human bypass vascular surgery. They further suggest that the model may be adaptable to investigate therapeutic strategies to prevent IH, an alteration which often compromise this surgery.

SUPPLEMENTARY MATERIAL

Supplementary material is available at ICVTS online.

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