Developing Human Umbilical Vein as Living Scaffolds for Vascular Tissue Engineering

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Abstract— **We have previously shown that endothelium-denuded human umbilical veins (HUV) are suitable scaffolds for vascular tissue engineering with contractile properties and endogenous prostacyclin release. This study established efficient methods to denude and seed HUV with allogeneic endothelial cells in a perfusion bioreactor in order to create small caliber vessel grafts. Stress-strain relationships and ultimate failure stresses were determined. The effects of a gas-based denudation method on contractile function, reductive capacity, and histology were evaluated. Finally, denuded HUV were seeded in a perfusion bioreactor to create small diameter vessel grafts. HUV displayed biphasic stress-strain relationships, but higher compliances compared to autologous vessels. Burst pressures extrapolated from the ring failure stresses was approx. 1000 mm Hg. Denudation by dehydration proved to be an effective method at gas flow rates of 60-120 ml/min. Endothelial cells were removed completely, whereas neither basal membranes nor functional properties were affected. Seeding of denuded HUV with endothelial cells in a perfusion bioreactor using suitable rotation patterns resulted in an even cell distribution and a confluent endothelium which was shear-resistant at flow rates up to 40 ml/min. These results indicate that the outlined procedures are suitable components of a protocol to turn HUV into small caliber vessel grafts.**

Keywords— **vascular tissue engineering, human umbilical vein, endothelium, seeding, HUVEC.**

I. Introduction

Western lifestyle is associated with a high incidence of vascular disorders like peripheral arterial occlusive disease and coronary artery disease. Many patients eventually require revascularization, using autologous vessels as bypass grafts. However, harvesting vessel grafts is impossible in a fraction of the patients due to prior removal, limb loss, or inadequacy as a consequence of concomitant diseases. Therefore artificial vessel grafts are being developed to alleviate this shortage of grafts.

Vessel grafts are expected to match the anastomosed vessels in terms of diameter and viscoelastic properties. Furthermore, the luminal surfaces are supposed to be non-thrombogenic, non-inflammatory and immunologically inert. Synthetic materials have not proven adequate for small caliber vessel grafts, in spite of their unquestionable successes in large-caliber vessel surgery. Tissue engineering is expected to provide better alternatives by combining biological or biocompatible scaffolds with autologous cells.

Umbilical cords are a cheap and abundant source of human vessels. Human umbilical veins (HUV) have been used in peripheral revascularizations for decades [1]. However, these vessels were fixed with glutaraldehyde, and hence acellular conduits without proper antithrombogenic properties and without any capability of remodeling. We have recently suggested to use endothelium-denuded HUV (den-HUV) as living scaffolds for vascular tissue engineering [2]. HUV were shown to contract to common vasoconstrictors, and they retain part of their contractile properties and of their reductive capacities after cryopreservation, which might allow banking of these vessels. They also release the antithrombotic compound prostacyclin even after denuding. These semi-finished products will have to be completed with a lining of autologous endothelial cells. They will also require modifications of the vessel wall to be suitable for arterial conditions, analogous to other published procedures. The current study explored several techniques to efficiently denude HUV and used a seeding procedure in a perfusion bioreactor to generate small caliber vessel grafts.

II. Materials and Methods

A. Vessel Procurement

Human umbilical cords were collected from uneventful pregnancies. Cords were stored in Krebs-Henseleit buffer supplemented with HEPES and antibiotics and analyzed within 24 hours of delivery [2]. HUV were dissected free from connective tissue and rinsed thoroughly with M199 to remove any blood clots.

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B. Tensile Testing

Stress-strain relationships and ultimate failure stresses were determined in a tensile testing rig (Inspekt Desk 50, Hegewald & Peschke). Vessel rings of 2 mm segment length were mounted on cylindrical supports. Rings were distended uniaxially at a constant speed of 10 mm per min until they failed.

C. Denudation

Based on an earlier report about denuding small caliber arteries [3], we perfused HUV with carbogen (95% oxygen, 5% carbon dioxide) at a flow rate of 120 ml/min for 10 min at room temperature while they were submerged in M199. Afterwards the vessels were thoroughly rinsed with M199 supplemented with 12% (w/v) dextran to remove cell debris. Short segments were removed from both ends for histological analysis to ensure complete removal of endothelium.

D. Contractile Properties

The responses of vessels to vasoactive compounds were tested in an organ bath (IOA-5300, FMI) as described previously [2]. In brief, vessel rings were mounted between two stainless steel hooks, the upper one being connected to a transducer which permitted isometric force measurements. The baths contained Krebs-Henseleit buffer which was continuously bubbled with a mixture of 5% oxygen and 5% carbon dioxide (balance nitrogen). After establishing a stable baseline, contractile responses to 150 mM KCl and to a concentration series of noradrenalin were recorded.

E. Tetrazolium Dye Reduction

Vessel segments were opened longitudinally and mounted in a vessel holder, luminal side facing up. Tetrazolium dye reduction, indicating the reductive capacities of the tissues, was determined according to standard protocols in the wells provided by the holder top.

F. Cell Culture

HUVEC and HSVEC were prepared enzymatically using previously published procedures [2]. HUVEC were cultured in M199 supplemented with 20% fetal calf serum and endothelial cell culture supplements. HSVEC were cultured in endothelial cell basal medium supplemented with 10% human serum and endothelial cell supplements. Cells were harvested in passages 1 or 2 for seeding experiments.

G. Perfusion System

Bioreactors (Fig. 1) consisted of medium reservoirs (1), neonatal membrane oxygenators (2), independent peristaltic pumps for perfusion and superfusion circulations (3,4), variable pulse dampeners (5), and vessel chambers (6). The medium consisted of M199 supplemented with 20% fetal calf serum. Oxygenators were flushed with a mixture of 20% oxygen and 5% carbon dioxide (balance nitrogen). Sterile ports allowed retrieval of medium samples for blood gas analysis. Media were replaced every other day. The vessel chambers were rotated in a computer-controlled fashion by stepper motors via timing belts.

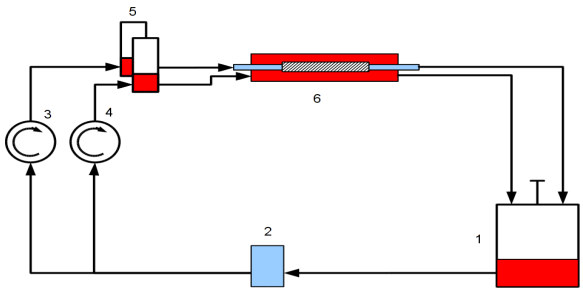

Fig. 1 Perfusion bioreactor

H. Seeding

Adherent cells were incubated with 40 µg/ml FITC-labeled Ulex europaeus lectin and 1 µg/ml Calcein AM for 1 hour at 37°C. Cells were then dislodged by trypsin and resuspended in M199 supplemented with 20% fetal calf serum and endothelial cell culture supplement at a density of approx. 5E6 cells/ml.

denHUV were perfused at 20 ml/min for at least one hour. Cell suspensions were injected into the vessels after stopping the perfusion. The superfusion circulation continued to run at 40ml/min during the seeding procedure.Vessels were rotated for 90° every three minutes for a total of 60 minutes. After that, perfusion was briefly started at 10ml/min to replenish the spent medium inside the vessels. Static incubation continued for another 60 minutes after which the perfusion was started at 10ml/min. After two hours of slow perfusion, the flow rate was incremented step-wise within 2 hours to 40 ml/min.

I. Histology

Short vessel segments were fixed in phosphate-buffered formaldehyde and embedded in paraffin. Thin sections were analyzed by H&E staining to visualize the general structure. Labeled endothelial cells were visualized under a UV microscope using appropriate band pass filters.

J. Statistics

Data are presented as mean±standard deviation and were compared using t-test or ANOVA as appropriate. Differences were accepted as significant if $p<0.05$.

III. Results

A. Tensile Properties

Vessel rings displayed biphasic stress-strain curves (Fig. 2). Stresses increased in an almost linear fashion until an approx. threefold distension. Further distension caused a more rapid increase of the measured stresses. Young's modulus at the steepest point of the curves was calculated as approx. 900 kPa. The rings failed at forces of 2.71±0.36 N (n=6). These values were extrapolated to a theoretical burst pressure of approx. 1000 mm Hg.

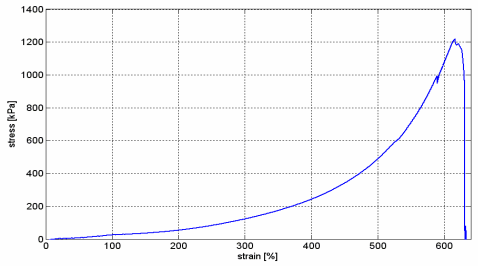

Fig. 2 Representative stress-strain curve of native HUV

B. Vessel Denudation

Most vessels tolerated a carbogen flow rate of 120 ml/min without any signs of distension. Histological analysis of denHUV showed a complete removal of endothelial cells without any changes of the remaining parts of the vessel wall (Fig. 3). SEM images show a structured basement membrane (Fig 4B).

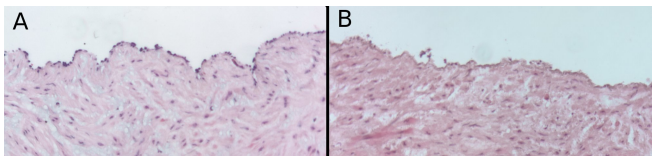

Fig. 3 Denudation by dehydration using a stream of carbogen. Tissue sections stained with hematoxylin and eosin (HE). (A) native control (B) denuded

C. Vessel Function after Denudation

Maximum contractile responses to KCl (HUV: 45.1+/-26.3 vs. denHUV: 37.6+/-18.8, n=6) and to serotonin (62.2+/-28.5 vs. 58.4+/-27.2) as well as reductive capacities (0.79+/-0.21 OD vs. 0.87+/-0.17) were not affected by denudation.

D. HUV Seeding

Several rotational patterns were tested empirically. Constant slow rotation (0.06 rpm) for 1 hour did not result in confluent endothelial cell layers. Intermittent rotation in 90° increments for 1 hour worked well with rotational speeds of 0.5 rpm and static phases of 3 to 5 minues, resulting in confluent endothelium as judged by SEM and histology (Fig. 4). Neoendothelium had a different appearance compared to native endothelium. In cross sections, cells appeared flattened and closely attached to the surface. Native endothelial cells were smaller and often showed a palisade-like appearance. SEM images indicated that native endothelium consisted of fairly small cells whereas neoendothelium was characterized by larger and elongated cells.

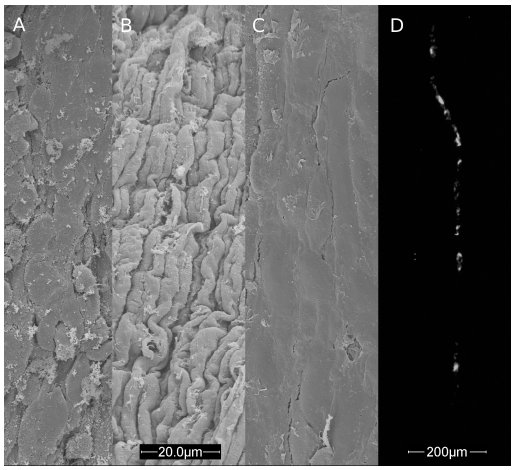

Fig. 4 (A) native HUV (B) denHUV (C) denHUV seeded with HUVEC (D) pre-labeled HUVEC visualized on the surface of seeded denHUV. A-C are en face scanning electron microscopy images, D is a cross-section fluorescent microscopy image

IV. Discussion

This study has investigated several aspects of HUV properties, proving their utility as living scaffolds for vascular tissue engineering. Most vascular tissue engineering protocols use synthetic or acellular scaffolds as starting materials. A major downside of many protocols is the long duration of up to almost one year [4], which is apparently necessary to build vessels from scratch, but which also precludes on-demand production of grafts. To the best of our knowledge, endothelium-denuded vessels have not been used in vascular tissue engineering so far, although these

half-finished products show a great potential to shorten the engineering process while still providing an immunologically inert graft.

Graft failures are often caused or promoted by a compliance mismatch between graft and anastomosed vessel. Native vessels show a higher compliance than synthetic materials. Stress-strain curves are also biphasic, allowing the vessels to adapt to physiological blood pressures effectively while limiting distension at high pressures. Our results demonstrate that HUV display stress-strain curves of a suitable shape, although the compliance is larger compared to other vessels commonly used for bypass grafting. Vessel grafts based on HUV will require a maturation phase which focuses on strengthening extracellular matrix.

Vessel denudation is a critical step in the production of grafts from HUV. Native endothelial cells must be removed completely without inflicting any damage to the remaining vessel wall. Pilot experiments had shown that methods based on proteolytic enzymes or distilled water are unsatisfactory as smooth muscle damage commences before endothelium removal is complete. In contrast, a gentle stream of gas was able to destroy endothelial cells, presumably by dehydration. Any remaining debris could be removed by thoroughly flushing denHUV. Therefore SEM images reveal a clean basal lamina, and endothelial cells were no longer detectable by histological methods. Functional analysis of denHUV demonstrated that smooth muscle function and metabolism were not affected by the denudation procedure. Although this study did not attempt to investigate this point in detail, it is tempting to speculate that the basal lamina is particularly well suited to limit the detrimental effects of a gas stream to the endothelium. Further histological analyses are required to corroborate this hypothesis.

We have previously shown that patches of denHUV can be seeded with HUVEC under static conditions [2]. The present study attempted to translate this into a procedure suitable to seed denHUV in a perfusion bioreactor. The main difference is the three-dimensional nature of the scaffold which requires rotation during seeding to achieve complete endothelial coverage. Constant rotation did not result in satisfactory cell adhesion, whereas slow rotation with intermittent static phases provided even cell distribution and complete coverage. Obviously, rotation is a prerequisite to distribute the cells over the entire luminal surface, but static phases seem to be essential to allow the cells to form sufficiently strong bonds to the vessel surface. Preliminary experiments indicated that seeding times beyond 1 hour do not noticeably improve the seeding results. The resulting endothelia were shear-resistant at flow rates up to 40ml/min. Currently experiments are under way to complete the tissue engineering process with a prolonged maturation phase which is supposed to increase the wall thickness to obtain better compliances and to adapt endothelial cells to even higher flow rates.

V. Conclusions

HUV appear well suited as starting materials for vessel grafts from a biomechanic point of view due to their biphasic stress-strain relationship, although the extracellular matrix must be further strengthened during a maturing phase to obtain appropriate compliances. This study has also outlined a reproducible procedure to denude HUV which does not affect the function of the remaining vessel wall. These denuded vessels can be seeded with autologous endothelial cells, resulting in a shear-resistant endothelium. Further work is required to establish a maturation phase in order to adapt the constructs to arterial conditions.

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