Evaluation of decellularized human umbilical vein (HUV) for vascular tissue engineering - comparison with endothelium-denuded HUV

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Abstract

Human umbilical vessels have been recognized as a valuable and widely available resource for vascular tissue engineering. Whereas endothelium-denuded human umbilical veins (HUV) have been successfully seeded with a patient-derived neoendothelium, decellularized vessels may have additional advantages due to their lower antigenicity. The present study investigated the effects of three different decellularization procedures on histological, mechanical, and seeding properties of HUV. Vessels were decellularized by detergent treatment (Triton X-100, sodium deoxycholate, IGEPAL-CA630), osmotic lysis (3M NaCl, distilled water), and by peroxyacetic acid treatment. In all cases nuclease treatments were required to remove residual nucleic acids. Decellularization resulted in a partial loss of fibronectin and laminin staining in the subendothelial layer and affected the appearance of elastic fibers. In addition to removing residual nucleic acids, nuclease treatment weakened all stainings and substantially altered surface properties as seen in scanning electron micrographs, indicating additional nonspecific effects. Detergent treatment and osmotic lysis caused failure stresses to decrease significantly. Although conditioned media prepared from decellularized HUV did not severely affect endothelial cell growth, cells seeded on decellularized HUV did not remain viable. This may be attributed to the partial removal of essential extracellular matrix components as well as to changes of surface properties. Therefore, decellularized HUV appear to require additional modifications in order to support successful cell seeding. Replacing the vessels' endothelium only should be considered as an alternative to decellularization when creating tissue engineered blood vessels with non-immunogenic luminal interfaces.

Keywords: vascular tissue engineering, small caliber graft, endothelium, biomechanics, human umbilical vein

1. Introduction

Coronary artery disease is a serious manifestation of cardiovascular disease and one of the leading causes of mortality in developed countries. Except for cases with a low number of stenoses, coronary artery bypass grafting (CABG) is the method of choice to extend the patients' lifespans (Serruys et al. 2009). Bypass grafting is commonly performed with autologous vessels such as internal mammary artery and saphenous vein. However, a considerable percentage of the eligible patients lack suitable vessels due to comorbidities, prior removal, or limb loss. Vessel grafts made from synthetic polymers have shown excellent results in large-diameter vessels but they have rarely been used in CABG due to the increased importance of the thrombogenicity of the luminal surface in small-caliber conduits (Zilla et al. 2007). Synthetic vessel grafts also show inferior viscoelastic properties compared to autologous vessels, causing a compliance mismatch at the anastomoses which eventually may develop into graft failure (Bordenave et al. 2005). Moreover, patency of saphenous vein grafts is approx. 60% after 10 years (Goldman et al. 2004) which often necessitates repeated CABG with a further limited choice of autologous grafts.

Tissue-engineered blood vessels (TEBV) have been suggested as vessel grafts for various purposes. They have been employed successfully as arteriovenous shunts for dialysis patients (McAllister et al. 2009), but to date their utility in peripheral or coronary revascularizations in humans remains to be shown. A variety of processes and approaches have been explored. Most of these combine acellular scaffolds with autolog-

ous cells, although some groups favor vessels created from sheets of cells which eliminates the need for a scaffold. A long duration, insufficient elastin formation, and insufficient mechanical properties hamper many current tissue engineering processes.

We have previously investigated human umbilical veins (HUV) as a source for TEBV and demonstrated that endothelium-denuded HUV can be seeded with autologous cells from CABG patients to form a confluent and flow-resistant neoendothelium (Hoenicka et al. 2007; Hoenicka et al. 2008; Hoenicka et al. 2011). The present study was undertaken to investigate the utility of decellularized HUV as scaffolds for vascular tissue engineering and to compare these two approaches.

2. Methods

2.1. Harvesting of umbilical veins

All experiments were approved by the ethics committee of University of Regensburg. Human umbilical cords were collected from term or near term pregnancies (gestational age at least 35 weeks) after obtaining written informed consent from the pregnant women. Donors with known infectious diseases like HIV or hepatitis were excluded. Cords were stored in Krebs-Henseleit buffer (KHB; NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, NaH_2PO_4 1.2 mM, $NaHCO_3$ 16.7 mM, dextrose 5.5 mM, $CaCl_2$ 1.2 mM) which contained penicillin (100 U/ml) and streptomycin (100 µg/ml, PAA, Pasching, Austria) as preservatives and HEPES (25 mM) to stabilize pH at 7.4. Umbilical veins were dissected free from Wharton's jelly and stored in KH for further processing.

2.2. Removal of endothelium

In order to prepare endothelium-denuded HUV, endothelial cells were destroyed by dehydration as described previously (Hoenicka et al. 2011). In brief, vessels were perfused with carbogen at 60 ml min^{-1} for 10 min and then rinsed thoroughly with culture medium. Endothelium removal was ascertained by histology and scanning electron microscopy of representative samples.

2.3. Decellularization procedures

Three methods to remove cells from the extracellular matrix of HUV were evaluated. These methods used different chemical or physical mechanisms to destroy and remove the cells, namely dissolving membranes by detergents, osmotic stress, and an oxidizing agent. In all procedures 1 ml of decellularization solution per cm of vessel length was used.

Method 1 was a modification of a protocol developed to prepare cell-free heart valves (Rieder et al. 2005). The decellularization solution had the following composition: 0.05% triton X-100, 0.05% sodium deoxycholate, and 0.05% IGEPAL-CA630. Samples were perfused with the decellularization solution and then placed in a screw-cap tube. The tubes were filled with decellularization solution and agitated on a rotating shaker.

Method 2 was a modification of osmotic lysis protocols commonly used in cell disruption. Vessels were perfused with 3 M NaCl and then incubated in screw-cap tubes with the same solution. The tubes were agitated at 4°C on a rotating shaker for 60 min. The same procedure was repeated with distilled water, 3 M NaCl, and again with distilled

water.

Method 3 was a modification of a procedure developed to isolate basement membrane components from several tissues (Brown et al. 2006). Vessels were perfused with a solution of peroxyacetic acid (PAA) in 4% ethanol. They were incubated in the same solution in screw-cap tubes on a rotating shaker at room temperature.

In all cases, the samples were perfused and washed thoroughly with phosphate-buffered saline to remove residual chemicals. Remaining traces of PAA were reduced by adding sodium bisulfite (0.5% w/v) to the washing solutions. Samples were stored in 70% ethanol for later analysis.

2.4. Removal of nucleic acids

Vessels treated with one of the above decellularization methods were further incubated in a mixture of DNAse I (100 µg ml^{-1}, Roche, Mannheim, Germany) and RNAse A (150 IU ml^{-1}, Sigma, Taufkirchen, Germany) in phosphate-buffered saline supplemented with 5 mmol L^{-1} $MgCl_2$ for 12h at 37°C on an orbital shaker to remove residual nucleic acids.

2.5. Histology and immunohistochemistry

Samples were fixed in phosphate-buffered paraformaldehyde solution (4%) and were subsequently embedded in paraffin. Thin sections were prepared in a microtome and mounted on slides. Tissues and their nuclei were visualized by hematoxylin and eosin (H&E, Chroma, Münster, Germany) staining using standard protocols. Collagen and elastin were stained with Sirius red (Sigma) and resorcin-fuchsin (Chroma), respectively. The following specific antibodies were used for immunohistochemistry: α-smooth

muscle cell actin (clone 1A4, monoclonal from mouse, Sigma), fibronectin (A0245, polyclonal from rabbit, Dako, Glostrup, Denmark), laminin (clone LAM-89, monoclonal from mouse, Sigma), versican (VCAN, LS-C25138, monoclonal from mouse, LifeSpan, Seattle, WA, USA), and von Willebrand factor (A0082, polyclonal from rabbit, Dako). Primary antibodies were labeled with appropriate biotinylated secondary antibodies from donkey (Jackson ImmunoResearch, Suffolk, UK) and visualized using the Vectastain Elite ABC kit (Vector, Burlingame, CA, USA) and diaminobenzidine (DAB) as chromogenic substrate.

2.6. Scanning electron microscopy

Samples were fixed in phosphate-buffered paraformaldehyde (2%) / glutardialdehyde (2.5%). Dehydrated samples were sputtered with gold according to standard protocols and analyzed in a LEO 1455 VP scanning electron microscope (Leo Electron Microscopy Ltd, Cambridge, UK).

2.7. Tensile testing

Stress-strain curves of vessel rings were recorded in a tensile testing rig as described previously (Hoenicka et al. 2011). In brief, rings of 2 mm segment length were mounted between two cylindrical supports which were moved apart at a constant speed of 10 mm min^{-1}, and force and displacement data were recorded continuously. At least four rings were tested per subject and treatment.

2.8. Human umbilical vein endothelial cell (HUVEC) culture

HUVEC were isolated from umbilical veins by limited proteolysis (Jaffe et al. 1973). Cell culture medium consisted of M199, 10% fetal calf serum (PAA), and endothelial cell culture supplement (Promocell, Heidelberg, Germany). Cultures were grown to confluence and subcultured using trypsin-EDTA. Cells of passages 1 or 2 were used for seeding experiments and for growth curves.

2.9. Seeding of HUVEC on decellularized and denuded vessels

To facilitate identification of seeded cells, HUVEC were labeled with Calcein AM, resuspended in culture medium, and adjusted to 5E6 cells mL^{-1}. The scaffolds were opened by a longitudinal cut, flattened carefully, and mounted in custom-built vessel holders. The upper half of the holders contained 5 wells with an area of 0.33 cm^2 and a volume of 200 µl, analogous to a 96-well plate. Cell suspensions were pipetted into the wells, resulting in seeding densities of 60000 cells cm^{-2}. Nonadherent cells were aspirated after 3 h, and fresh culture medium was added to the wells. Controls were seeded on 96-well tissue culture plates.

Seeding results were evaluated after 1, 3, and 6 days of static culture in a cell culture incubator. The holders were disassembled and the vessels transferred to microscopic slides. Cell distribution was recorded by fluorescence imaging (Leica DMRBE, Leitz, Wetzlar, Germany). Samples were further processed for histology and scanning electron microscopy.

2.10. Determination of tetrazolium dye reduction

Cellular reduction of the chromogenic substrate 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega, Madison, WI, USA) to a brownish dye was determined as described previously (Hoenicka et al. 2007) and was used to monitor the metabolic activity of seeded cells on decellularized scaffolds.

2.11. Growth inhibition of endothelial cell cultures

The influence of residual chemicals introduced by the decellularization procedures on the growth of endothelial cell cultures was assessed by constructing HUVEC growth curves. After decellularization of HUV was finished including the washing steps described above, the vessels were incubated in cell culture medium for 1 or 5 days on a rotating shaker at 4°C to create conditioned media.

Cells from four independent HUVEC cultures (i.e. isolated from four umbilical cords) were seeded at 2000 cells per well on 96-well plates using either fresh cell culture medium as a control or one of the conditioned media. Cells were trypsinized and counted in an automated cell counter (CASY, Roche, Basel, Switzerland) every other day starting on day 3, and tetrazolium dye reduction was determined. Media were replenished with fresh and conditioned media, respectively, on days 4 and 8.

2.12. Data analysis and statistical procedures

Data are presented as mean±standard deviation. n refers to the number of patients.

Growth curves were compared by a two-way repeated measurements ANOVA, followed by Holm-Sidak post-test. Differences were assumed to be significant if the error probability p was less than 0.05.

2.13. Drugs, chemicals, and reagents

All chemicals were of analytical grade and were obtained from Sigma or from Merck (Darmstadt, Germany) unless noted otherwise.

3. Results

3.1. Optimization of decellularization procedures

The effects of decellularization procedures on various components of vessel walls were assessed by histochemistry and immunohistochemistry (Fig. 1). Stainings of native HUV were comparable to adult vessels with the notable exception of laminin. In adult vessels like radial artery, laminin staining is restricted to the subendothelial layer (not shown), whereas in HUV this protein was detectable throughout the entire smooth muscle layer. Elastic fibers as indicated by resorcin staining were limited mainly to the subendothelial layer and a few additional layers within the luminal side of the smooth muscle layer. Versican was limited to the subendothelial layer as well.

The parameters of decellularization procedures and of nuclease treatment were optimized empirically according to the matrix outlined in Table 1. Conditions were adjusted to minimize intracellular stainable materials while maintaining most of the extracellular matrix components. The following description refers to these optimized conditions.

Decellularization by detergents had a readily noticeable effect on most stainings. There was less H&E stainable material and less collagen as indicated by sirius red. However, other components like smooth muscle actin, elastin, and fibronectin appeared to be well preserved. The other decellularization methods showed less impact on vessel wall proteins. None of the methods was able to remove cell nuclei entirely, and nuclear staining was still well preserved after detergent treatment.

Nuclease treatment successfully removed the remainder of nuclei after osmotic lysis and PAA treatment, whereas dergent-treated vessels still showed low traces of nuclear material (Fig. 2). However, nuclease treatment had noticeable effects besides removal of nuclear material. Detergent-treated vessels and vessels decellularized by osmotic lysis showed weaker stainings of almost all parameters after nuclease treatment. Specifically, detergent-treated vessels lost versican and fibronectin staining almost completely, and both elastin and collagen staining were further weakened. In contrast, PAA-treated HUV retained most of the staining even after nuclease treatment. Most notably, the structural proteins elastin and collagen as well as two proteins from the subendothelial layer, fibronectin and versacin, were well preserved, whereas laminin staining was reduced.

Luminal surfaces of HUV were analyzed by scanning electron microscopy (Fig. 3). Native HUV displayed a confluent endothelium composed of tightly packed endothelial cells. Endothelium-denuded HUV showed a peculiar surface structure that appears to reflect the organization of smooth muscle cells underneath the basal membrane. Decellularized HUV were apparently not entirely free of endothelial cell debris as the surfaces

did not resemble those of endothelium-denuded vessels. Regardless of the decellularization procedure, nuclease treatment caused further changes in the surface appearances. HUV decellularized by PAA or by osmotic lysis closely resembled endothelium-denuded vessels, whereas the surfaces of HUV decellularized by detergents were still covered by debris.

3.2. Mechanical Properties

Ultimate failure strengths of native HUV rings amounted to 2.39±0.42 N. Decellularization by osmotic lysis or by PAA did not alter ultimate failure strengths, whereas vessels treated with detergents appeared to have an increased ultimate failure strength (Fig. 4). Removal of nucleic acids in vessels decellularized by osmotic lysis resulted in significantly lower ultimate failure strengths compared to native controls. Nuclease treatment also weakened vessels decellularized by detergents significantly (ANOVA, $p<0.001$, $n=4$).

3.3. Influence of conditioned media on endothelial cell growth

Conditioned media, created by washing decellularized vessel segments in HUVEC growth medium for one or five days, were assessed for their capability to promote HUVEC proliferation in comparison to untreated growth medium (Fig. 5). None of the conditioned media negatively affected cell growth compared to the untreated growth medium. Conversely, medium conditioned with vessels denuded by PAA treatment caused significantly higher cell counts on days 7 through 13 compared to the other treatments, independent of the incubation time of the vessels in the medium (ANOVA,

$p<0.001$, $n=4$).

There was a small but significant inhibitory effect of some of the conditioned media on tetrazolium dye reduction (Fig. 6). Cells grown in media equilibrated with vessels decellularized by detergents and by osmotic lysis reached a lower maximum reductive capacity, indicating a minor but noticeable inhibitory effect of these media on cell function, assuming that the cell counts did not differ in these samples as described above (ANOVA, $p<0.001$, $n=4$).

3.4. Seeding results

HUVEC were seeded on the luminal surface of decellularized HUV and incubated under static conditions for 1, 3, and 6 days in a cell culture incubator. Control cells of the same cultures were grown on microplates. Scanning electron microscopy revealed that the seeded cells had no tendency to spread out and flatten on the surface (Fig. 7). Only few cells were observed that appeared flat and showed lamellipodia (Fig. 7 A), but these were not able to make contacts with neighboring cells and hence did not form a confluent neoendothelium. Most cells retained their spherical form. Starting on day 3, some of the cells appeared porous, indicating substantial structural damage. On day 6, essentially all cells appeared dead. Cells seeded on decellularized HUV reduced significantly less MTS on day 1 compared to control cells, and displayed a decrease over time (ANOVA, $p<0.001$). Control cells seeded on microplates showed significant increases of MTS reduction over time (ANOVA, $p<0.001$), indicating cell proliferation (Fig. 8). These results were independent of the method used for decellularization.

4. Discussion

The unique combination of collagen, elastin, and a variety of other accessory proteins is responsible for the nonlinear elastic properties of blood vessels, a feature which has been preserved or even re-invented throughout phylogeny (Shadwick 1999). Blood vessels are sufficiently elastic to conduct the pulsatile flow generated by the heart but sufficiently strong to resist irreversible distension during pressure peaks. An ideal vessel graft should mimick these properties. Therefore the use of vessels as the starting material of vascular tissue engineering is essentially a straightforward approach. Among others, xenogenic arteries (Teebken et al. 2001; Tamura et al. 2003) and veins (Lü et al. 2009) have been used as vascular scaffold source. Allogeneic human arteries have been used successfully for bypass grafting after seeding autologous endothelial cells on the luminal surface (Lamm et al. 2001). Recently, we (Hoenicka et al. 2007; Hoenicka et al. 2011) and other groups (Daniel et al. 2005; Gui et al. 2009) have started to develop umbilical vessels as a scaffold source for vascular grafts. Umbilical cords are easy to obtain without ethical concerns, as they are usually discarded post partum. Umbilical vessels do not possess valves or branches, thus flow direction is unimportant and ligation of side branches is not required as in other vessels. The walls of umbilical vessels are simplified in comparison to adult veins or arteries in that they lack a clearly defined adventitia. Both Wharton's jelly and the cord amnion appear to provide elastic properties to compensate for the lack of that layer. In contrast to umbilical arteries, the veins possess considerable amounts of elastin (Ferguson and Dodson 2009) (see also Fig. 2) and thus show viscoelastic properties closer to adult vessels. We have previously

demonstrated that umbilical veins can be efficiently endothelium-denuded and re-endothelialized in a perfusion bioreactor using human saphenous vein endothelial cells (Hoenicka et al. 2011). Although allogeneic vessels seeded with an autologous endothelium have been used successfully as bypass grafts without immunosuppression (Lamm et al. 2001), the immunogenic properties of denuded umbilical veins have not yet been explored in sufficient detail. Decellularization removes a considerable amount of immunogenic structures. Therefore the present study investigated the utility of decellularized umbilical veins for vascular tissue engineering.

Decellularization techniques have been devised for a variety of cardiovascular structures, e.g. the above mentioned blood vessels, heart valves (Cebotari et al. 2002; Rieder et al. 2005), and entire hearts (Ott et al. 2008). These reports used detergents or other chemical agents to disrupt and remove cells from the extracellular matrix, optionally followed by a nuclease treatment to remove remainders of nucleic acids. However, some of the inherently cytotoxic substances may prove difficult to remove entirely, so other disrupting methods using physical principles were tested as well (Gilbert et al. 2006).

The present study compared three independent decellularization procedures in order to find one which best preserves the extracellular matrix while securely removing cells and nuclei. The first method used detergents, the second method osmotic lysis, and the third one used an oxidizing acid. Histological and immunohistological evaluation of the decellularized vessels indicated that none of the methods was sufficient to remove nucleic acids entirely, although all remaining nuclei were clearly defunct. Laminin showed an

unusual distribution throughout the vessel wall. In most adult vessels laminin is restricted to the subendothelial layer. This was confirmed in our lab using control sections of radial arteries and saphenous veins (not shown). Detergent treatment caused the most obvious changes in all staining methods. In particular, elastin and collagen stained weaker, and there was less H&E-reactive material compared to the other methods. In order to remove the remaining nucleic acids, the decellularized samples were treated with nucleases. After this treatment, both vessels decellularized by detergents and by osmotic lysis showed a remarkable loss of extracellular proteins, in particular fibronectin, laminin, elastin, collagen, and versican. The latter is virtually absent from detergent-treated vessels. On the other hand, PAA-treated vessels did not show a loss of stainable material except for a weaker laminin staining. These results indicate that there may have been some remaining activity of endogenic proteases which were still active during the nuclease incubation. PAA apparently oxidized these enzymes and rendered them inactive. A similar pattern was observed when the ultimate failure strength was determined. Nuclease treatment did not have any effect on PAA-decellularized vessels. On the other hand, the strength of vessels treated with one of the other methods appeared to decrease after nuclease treatment. Preliminary experiments with broadband protease inhibitors (PMSF and EGTA, not shown) did not improve these results, although a wider panel of inhibitors would be required to unequivocally prove the participation and type of endogenous proteases.

Scanning electron microscopy revealed that the luminal surfaces of vessels decellularized with detergents was not entirely free of debris even after nuclease treatment. Ves-

sels decellularized with the other methods showed a structure comparable to endothelium-denuded vessels only after nuclease treatment. We therefore conclude that nuclease treatment is not only required to remove nucleic acids, but it also helps to obtain a cleaner scaffold. Gui et al. reported a similar effect of a serum incubation step to complete vessel decellularization (Gui et al. 2010).

Growth curves of endothelial cells in conditioned media were recorded to ensure that the decellularized and nuclease-treated vessels were washed sufficiently and did not release any cytotoxic substances into the medium. Similar techniques have previously been used to estimate the effects of residual detergents after heart valve decellularization (Cebotari et al. 2010). Two different incubation times during conditioning were chosen to distinguish immediate from slow-acting growth inhibiting effects, if any. The results clearly indicate that there was no growth inhibition to a degree that would explain the results of the seeding experiments. Tetrazolium dye reduction in cell cultures is influenced both by cell numbers and by reductive capacities of the cells. The growth curves provided both cell counts and dye reduction data and thus allowed to discriminate between both mechanisms. The small but significant promotion of cell growth seen in the presence of medium conditioned in vessels decellularized with PAA is hard to explain, especially as the tetrazolium dye reduction was not different from the controls. Conditioned media taken from vessels decellularized with detergents or by osmotic lysis caused a small but significant decrease in tetrazolium dye reduction. However, the magnitude of this effect did not indicate that cell attachment and growth might be seriously affected. While this assay demonstrated that the scaffolds did not release any diffusable

cytotoxic substances, there still may have been some additional growth inhibition by compounds closely attached to the scaffold.

Regardless of the lack of serious growth-inhibiting effects of conditioned media, seeded endothelial cells were not able to spread on the surface and create a confluent monolayer. Interestingly, limited proteolysis with trypsin/EDTA was not able to remove the majority of cells as judged under a fluorescence microscope and concomitant cell counting of the detached cells (not shown). Apparently the cells did make an initial contact but were unable to flatten. Two mechanisms may be responsible. First, adhesion and growth of many cells is influenced by surface structures and by mechanical properties of the scaffold. There is ample evidence that surface topology influences cell behaviour (Curtis and Wilkinson 1997). In a study using scaffolds with increasing compressive moduli due to increased crosslinking, osteoblast adhesion and growth was shown to be proportional to substrate stiffness (Haugh et al. 2011). Second, there may be a lack of crucial extracellular matrix proteins which the cells require to attach successfully. Histology of the decellularized samples showed that matrix proteins considered crucial for cellular adhesion were affected by the decellularization procedures. Scaffolds decellularized by detergent treatment or by osmotic lysis showed considerably weaker staining for a variety of matrix proteins, especially fibronectin, laminin, elastin, collagen, and versican, especially after nuclease treatment. Those decellularized by PAA treatment showed a particularly weak laminin staining, whereas other markers were better preserved compared to the other decellularization methods. PAA is both an acid and a strong oxidizing agent. As it is used for crosslinking polymers, similar effects should be

expected in biological polymers. This might in fact alter the properties of proteins without necessarily affecting their antibody recognition.

These results are in stark contrast to the previously published seeding of endothelium-denuded HUV. These studies demonstrated that denuded HUV can be seeded under similar conditions as in the present study, resulting in a confluent endothelium (Hoenicka et al. 2007). Denuded HUV, which have the same failure strength as native vessels, can also be seeded in a perfusion bioreactor and develop a confluent and flow-resistant neoendothelium (Hoenicka et al. 2011). Other groups have investigated seeding of decellularized umbilical vessels as well. Detergent-decellularized human umbilical arteries supported endothelial cell attachment only after applying a fibronectin coating (Gui et al. 2009). However, Daniel, Abe, and McFetridge (2005) demonstrated that detergent-decellularized HUV supports the attachment and migration of smooth muscle cells. Endothelial cell seeding was not reported though. We conclude from these studies that endothelium-denuded HUV provide a superior surface for endothelial cell attachment without the need to supplement additional matrix proteins.

5. Conclusion

This study compared three different decellularization methods and applied these to HUV, trying to obtain scaffolds suitable for vascular tissue engineering. However, none of the methods was able to provide a scaffold which supported endothelial cell attachment and growth without a feeder layer of other vascular cells or without precoating with extracellular matrix proteins. This indicates that other approaches, especially the use of endothelium-denuded vessels which we had reported previously, should be considered

as useful alternatives to decellularization.

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References

Bordenave L, Fernandez P, Rémy-Zolghadri M, Villars S, Daculsi R, Midy D. 2005, In vitro endothelialized ePTFE prostheses: clinical update 20 years after the first realization, *Clin Hemorheol Microcirc*, **33**: 227-234

Brown B, Lindberg K, Reing J, Stolz DB, Badylak SF. 2006, The basement membrane component of biologic scaffolds derived from extracellular matrix, *Tissue Eng*, **12**: 519-526

Cebotari S, Mertsching H, Kallenbach K, Kostin S, Repin O, Batrinac A, Kleczka C, Ciubotaru A, Haverich A. 2002, Construction of autologous human heart valves based on an acellular allograft matrix, *Circulation*, **106**: I63-I68

Cebotari S, Tudorache I, Jaekel T, Hilfiker A, Dorfman S, Ternes W, Haverich A, Lichtenberg A. 2010, Detergent decellularization of heart valves for tissue engineering: toxicological effects of residual detergents on human endothelial cells, *Artif Organs*, **34**: 206-210

Curtis A, Wilkinson C. 1997, Topographical control of cells, *Biomaterials*, **18**: 1573-1583

Daniel J, Abe K, McFetridge PS. 2005, Development of the human umbilical vein scaffold for cardiovascular tissue engineering applications, *ASAIO J*, **51**: 252-261

Ferguson VL, Dodson RB. 2009, Bioengineering aspects of the umbilical cord, *Eur J Obstet Gynecol Reprod Biol*, **144 Suppl 1**: S108-S113

Gilbert TW, Sellaro TL, Badylak SF. 2006, Decellularization of tissues and organs, *Biomaterials*, **27**: 3675-3683

Goldman S, Zadina K, Moritz T, Ovitt T, Sethi G, Copeland JG, Thottapurathu L, Krasnicka B, Ellis N, Anderson RJ, Henderson W, VA Cooperative Study Group #207/297/364. 2004, Long-term patency of saphenous vein and left internal mammary artery grafts after coronary artery bypass surgery: results from a Department of Veterans Affairs Cooperative Study, *J Am Coll Cardiol*, **44**: 2149-2156

Gui L, Chan SA, Breuer CK, Niklason LE. 2010, Novel utilization of serum in tissue decellularization, *Tissue Eng Part C Methods*, **16**: 173-184

Gui L, Muto A, Chan SA, Breuer CK, Niklason LE. 2009, Development of decellularized human umbilical arteries as small-diameter vascular grafts, *Tissue Eng Part A*, **15**: 2665-2676

Haugh MG, Murphy CM, McKiernan RC, Altenbuchner C, O'Brien FJ. 2011, Crosslinking and mechanical properties significantly influence cell attachment, proliferation, and migration within collagen glycosaminoglycan scaffolds, *Tissue Eng Part A*, **17**: 1201-1208

Hoenicka M, Jacobs VR, Huber G, Schmid F, Birnbaum DE. 2008, Advantages of human umbilical vein scaffolds derived from cesarean section vs. vaginal delivery for vascular tissue engineering, *Biomaterials*, **29**: 1075-1084

Hoenicka M, Lehle K, Jacobs VR, Schmid FX, Birnbaum DE. 2007, Properties of the human umbilical vein as a living scaffold for a tissue-engineered vessel graft, *Tissue Eng*, **13**: 219-229

Hoenicka M, Schrammel S, Bursa J, Huber G, Bronger H, Schmid C, Birnbaum DE. 2011, Development of Endothelium-Denuded Human Umbilical Veins as Living Scaffolds for Tissue-Engineered Small Caliber Vascular Grafts, *J Tissue Eng Regen Med*, (in press)

Jaffe EA, Nachman RL, Becker CG, Minick CR. 1973, Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria, *J Clin Invest*, **52**: 2745-2756

Lamm P, Juchem G, Milz S, Schuffenhauer M, Reichart B. 2001, Autologous endothelialized vein allograft: a solution in the search for small-caliber grafts in coronary artery bypass graft operations, *Circulation*, **104**: I108-I114

Lü W, Zhang M, Wu Z, Hu T. 2009, Decellularized and photooxidatively crosslinked bovine jugular veins as potential tissue engineering scaffolds, *Interact Cardiovasc Thorac Surg*, **8**: 301-305

McAllister TN, Maruszewski M, Garrido SA, Wystrychowski W, Dusserre N, Marini A, Zagalski K, Fiorillo A, Avila H, Manglano X, Antonelli J, Kocher A, Zembala M, Cierpka L, de la Fuente LM, L'Heureux N. 2009, Effectiveness of haemodialysis

access with an autologous tissue-engineered vascular graft: a multicentre cohort study, *Lancet*, **373**: 1440-1446

Ott HC, Matthiesen TS, Goh S, Black LD, Kren SM, Netoff TI, Taylor DA. 2008, Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart, *Nat Med*, **14**: 213-221

Rieder E, Seebacher G, Kasimir M, Eichmair E, Winter B, Dekan B, Wolner E, Simon P, Weigel G. 2005, Tissue engineering of heart valves: decellularized porcine and human valve scaffolds differ importantly in residual potential to attract monocytic cells, *Circulation*, **111**: 2792-2797

Serruys PW, Morice M, Kappetein AP, Colombo A, Holmes DR, Mack MJ, Ståhle E, Feldman TE, van den Brand M, Bass EJ, Van Dyck N, Leadley K, Dawkins KD, Mohr FW, SYNTAX Investigators. 2009, Percutaneous coronary intervention versus coronary-artery bypass grafting for severe coronary artery disease, *N Engl J Med*, **360**: 961-972

Shadwick RE. 1999, Mechanical design in arteries, *J Exp Biol*, **202**: 3305-3313

Tamura N, Nakamura T, Terai H, Iwakura A, Nomura S, Shimizu Y, Komeda M. 2003, A new acellular vascular prosthesis as a scaffold for host tissue regeneration, *Int J Artif Organs*, **26**: 783-792

Teebken OE, Pichlmaier AM, Haverich A. 2001, Cell seeded decellularised allogeneic matrix grafts and biodegradable polydioxanone-prostheses compared with arterial autografts in a porcine model, *Eur J Vasc Endovasc Surg*, **22**: 139-145

Zilla P, Bezuidenhout D, Human P. 2007, Prosthetic vascular grafts: wrong models, wrong questions and no healing, *Biomaterials*, **28**: 5009-5027

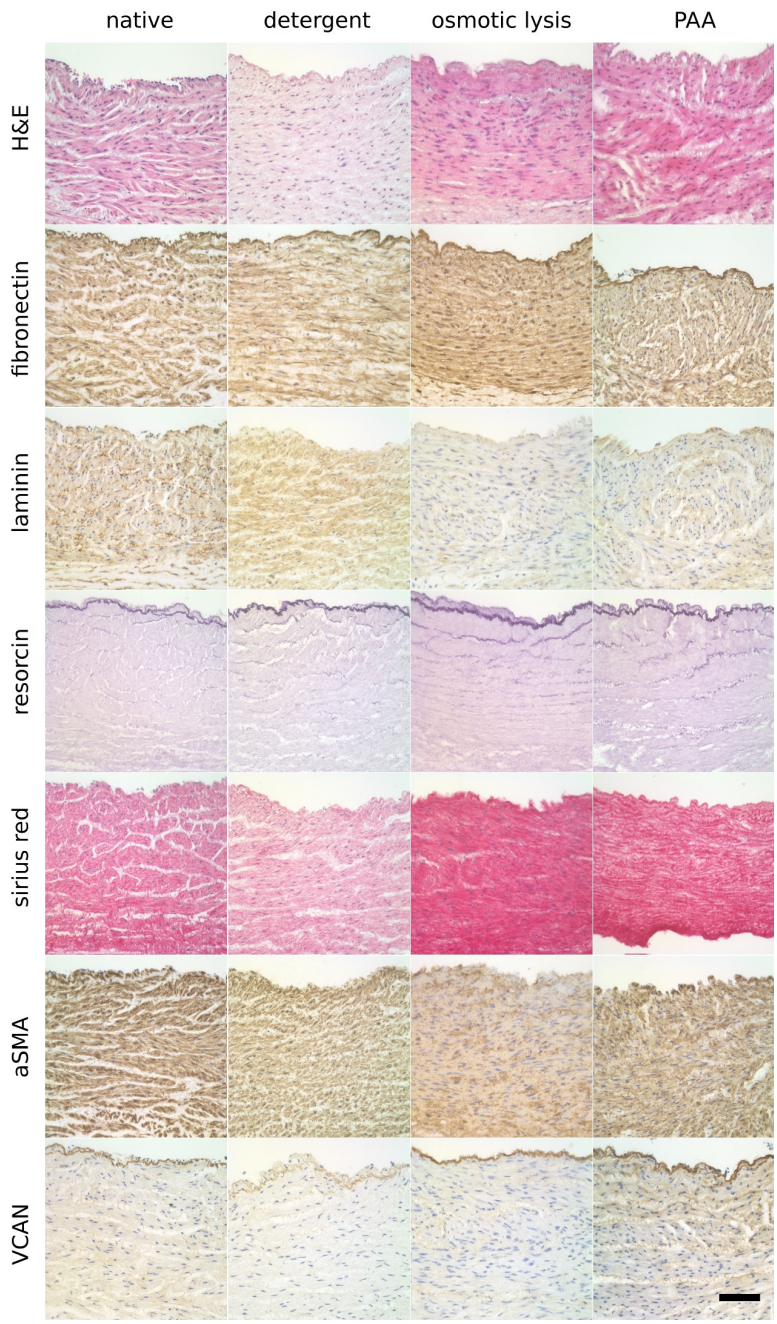

Fig. 1: Histological evaluation of HUV decellularization. H&E: general morphology. Fibronectin, Laminin, aSMA, VCAN: brown stain by DAB indicates specific antibody binding at fibronectin, laminin, α-smooth muscle actin, and versican epitopes, respectively. Resorcin: dark purple color indicates elastic fibers. Sirius Red: intense red color indicates collagen. Images are representative for 3-4 independent sample preparations. Bar indicates 100 µm.

Fig. 2: Histological evaluation of HUV decellularization followed by nuclease treatment. See Fig. 1 for explanations. Images are representative for 3-4 independent sample preparations. Bar indicates 100 µm.

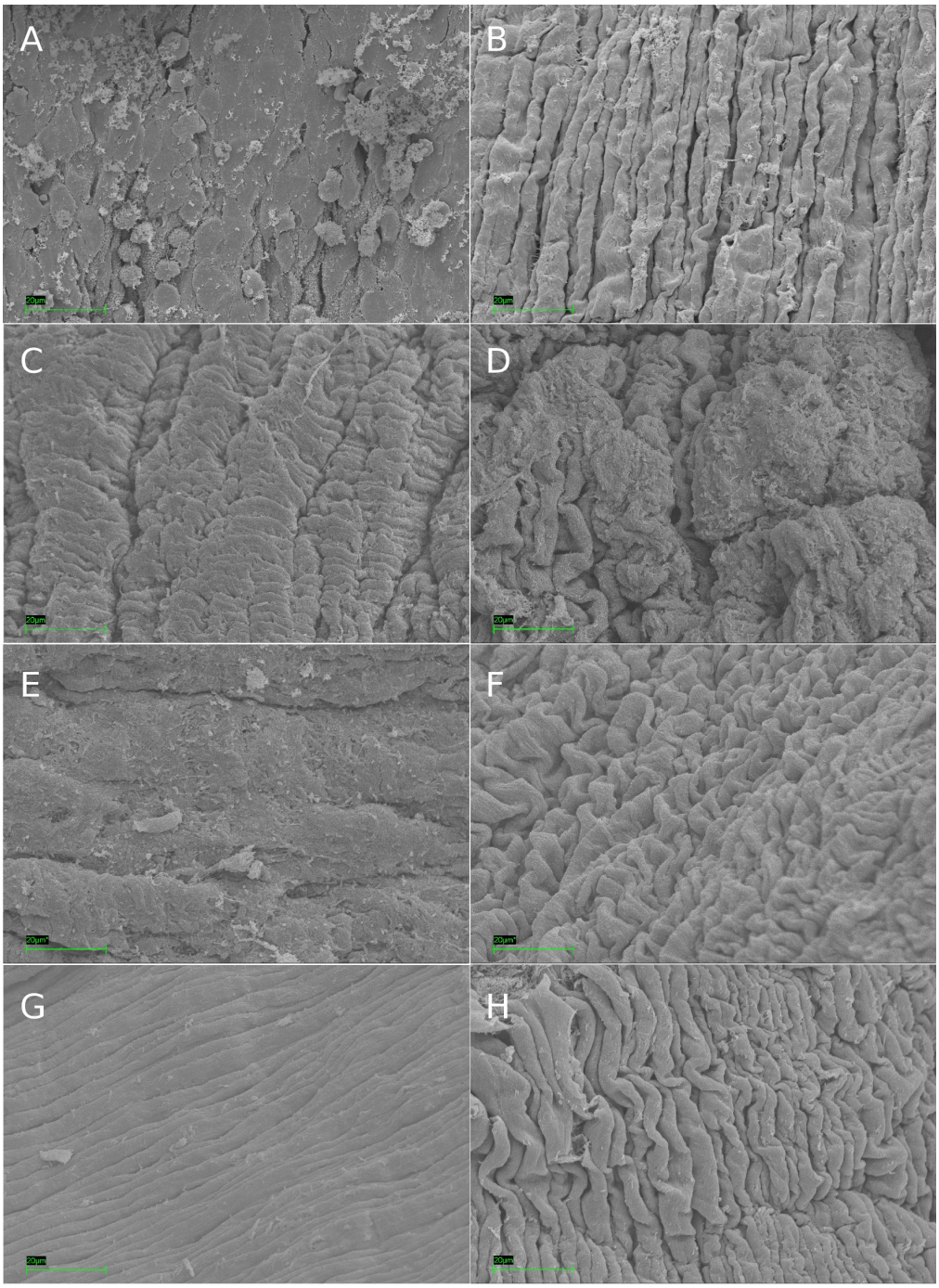

Fig. 3: Scanning electron microscopy images of HUV luminal surfaces. (A) native HUV; (B) endothelium-denuded HUV; (C) HUV decellularized by detergents before and (D) after nuclease treatment; (E) HUV decellularized by osmotic lysis before and (F) after nuclease treatment; (G) HUV decellularized by PAA before and (H) after nuclease treatment. Images are representative for 3 independent preparations per condition. Bars indicate 20 µm.

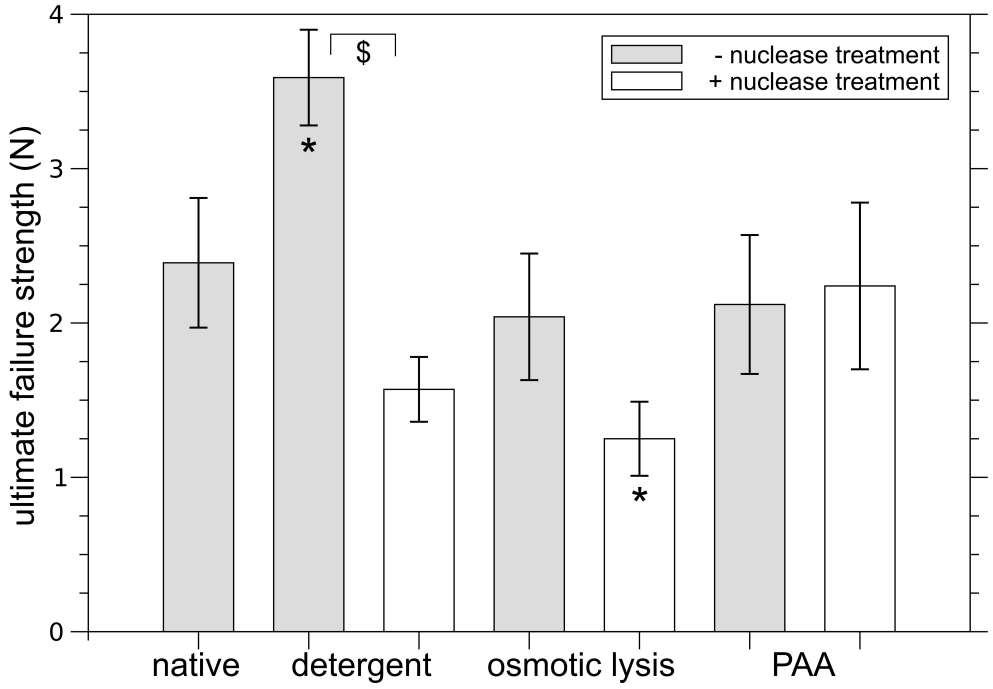

Fig. 4: Tensile testing of native and decellularized HUV. Filled bars, without nuclease treatment. Open bars, with nuclease treatment. * significantly different from native controls (ANOVA, $p<0.001$, $n=4$). $ significantly different (ANOVA, $p<0.001$).

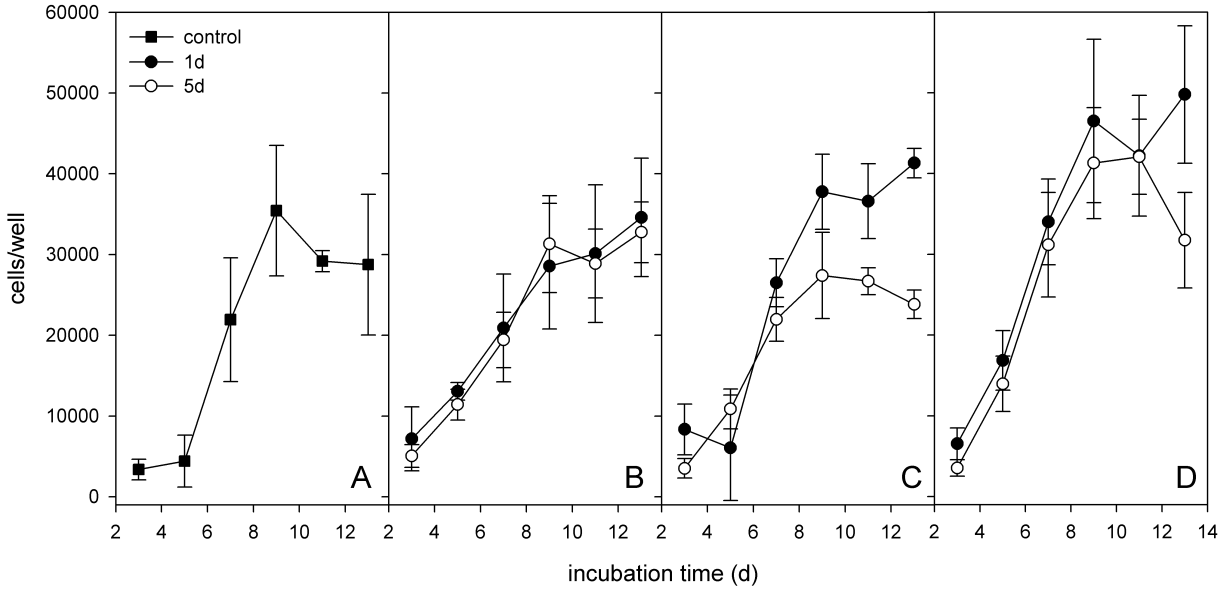

Fig. 5: Effect of conditioned media on HUVEC proliferation (cell counts). (A) control. (B) detergent (C) osmotic lysis (D) PAA. Media conditioned in the presence of PAA-treated HUV caused a small but significant increase of cell numbers compared to the control (ANOVA, $p<0.001$, $n=4$).

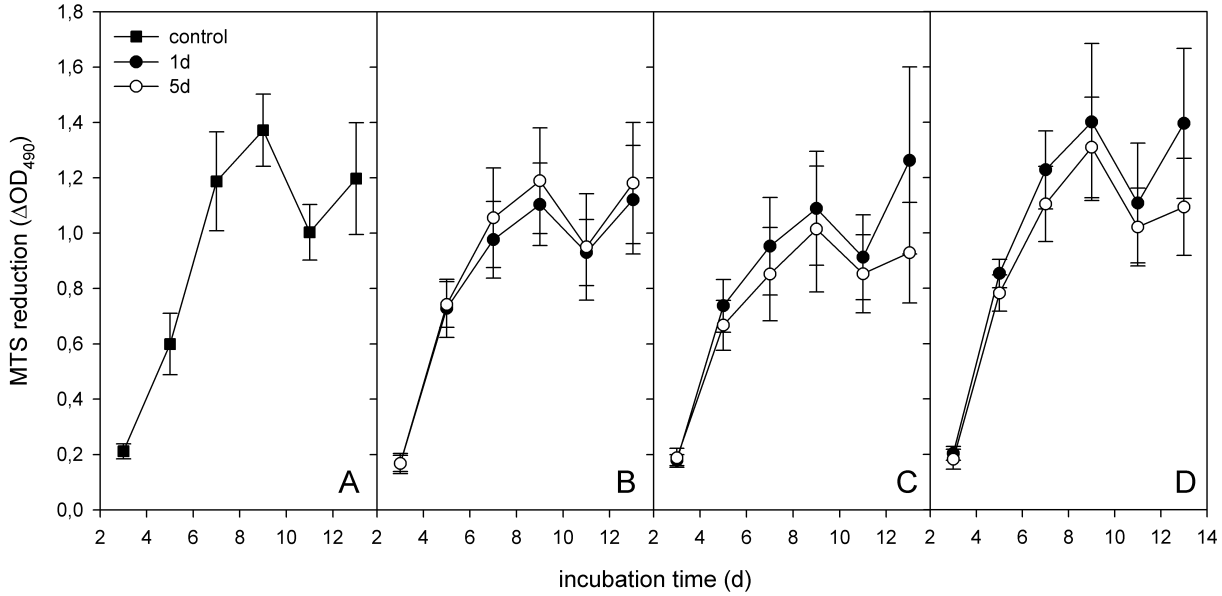

Fig. 6: Effect of conditioned media on HUVEC proliferation (tetrazolium dye reduction). (A) control. (B) detergent (C) osmotic lysis (D) PAA. Media conditioned in the presence of vessels decellularized with detergents or by osmotic lysis caused a small but significant decrease of maximum reductive capacities (ANOVA, $p<0.001$, $n=4$).

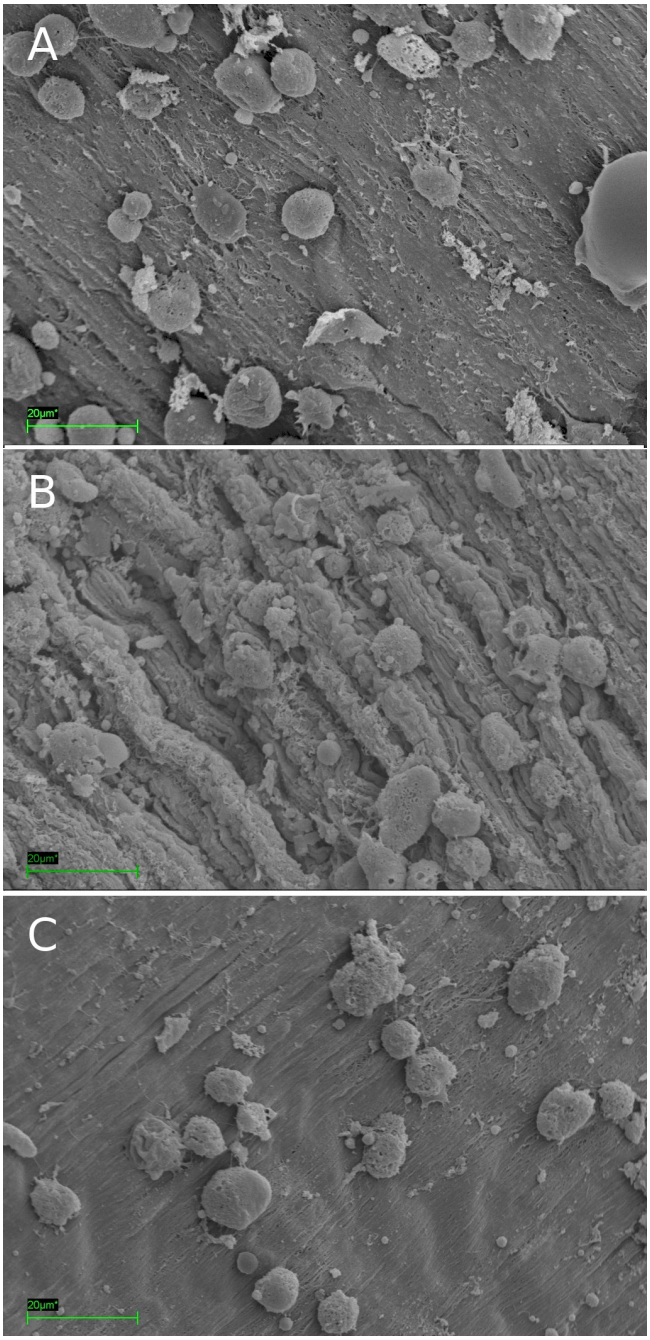

Fig. 7: Seeding of decellularized HUV. The scaffolds shown here were decellularized with PAA but are representative for the other decellularization methods as well. HUVEC were seeded on day 0 at a density of 60000 cells cm^{-2}. Scanning electron micrographs were prepared on (A) day 1, (B) day 3, and (C) day 6. Note the increasing number of porous cells. Images are representative for 3 independent seeding experiments per decellularization method. Bars indicate 20 µm.

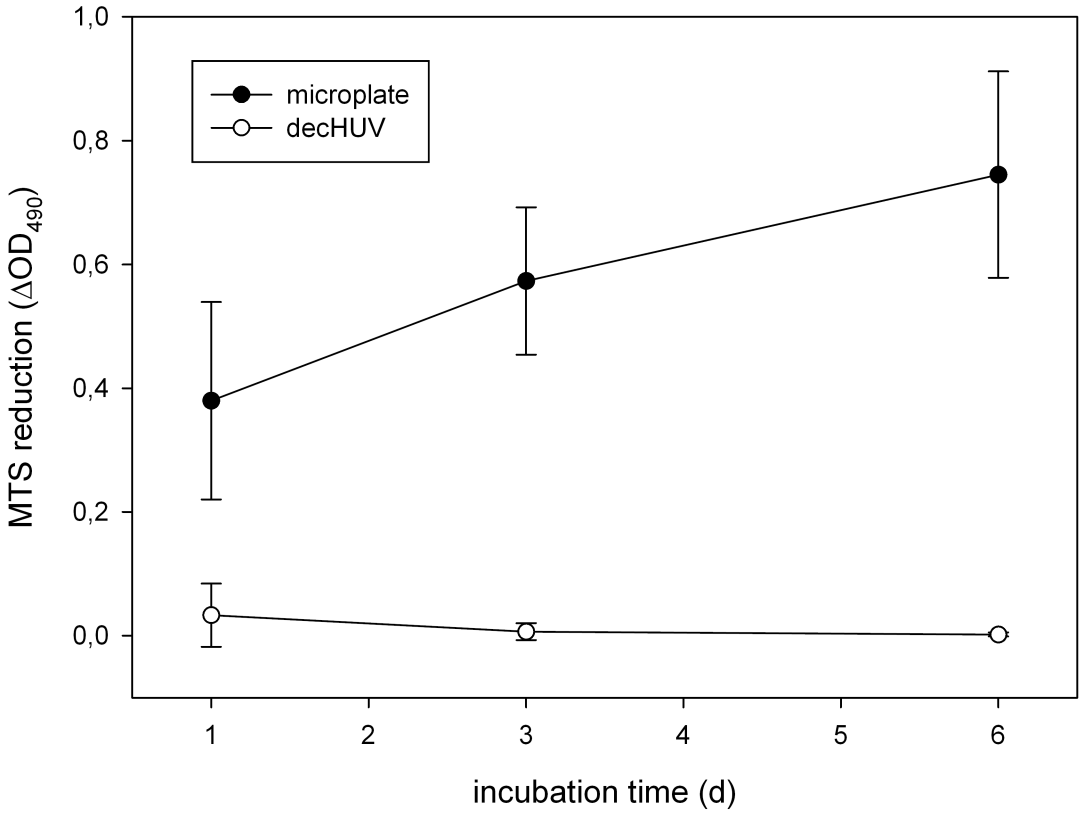

Fig. 8: Growth curves of HUVEC as determined by tetrazolium dye reduction. Cells were seeded at a density of 60000 cells cm^{-2} on day 0 on either cell-culture grade microwell plates (filled circles) or on decellularized HUV (decHUV, open circles). Data were collected from 6 independent seeding experiments involving 4 to 14 vessel samples and one microwell control per experiment. HUV data were averaged without regard to the decellularization method used as no differences between these methods were apparent. MTS reduction of cells seeded on microwell plates increased significantly over time, and MTS reduction of cells seeded on decellularized HUV decreased significantly over time (ANOVA, $p<0.001$).

	concentration	temperature	incubation time
detergent	full strength, **0.5x**	4°C, **37°C**	8, 12, **24**, 48h
osmotic lysis	**full strength**	**4°C**	**2 repeats**, 3 repeats
PAA	0.1%, 0.3%, **1%**	**RT**	15 min, 30 min, **1 h**, 2 h, 16 h, 24 h
nuclease treatment	**full strength**	**37°C**	4 h, 8 h, **12 h**

Table 1: Parameters of empirical optimization of decellularization and nuclease treatment conditions. The conditions in boldface were used for all subsequent experiments.