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Isolation of Primary Endothelial and Stromal Cell Cultures of the Corpus Cavernosum Penis for Basic Research and Tissue Engineering

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Abstract

Objectives: Primary cell cultures derived from the corpus cavernosum are frequently used as in vitro models to define cellular mechanisms involved in erectile function. However, previous studies often lack detailed isolation protocols or a precise characterisation of the culture composition excluding especially contaminating fibroblasts. This study aimed at critically analysing and reproducing reported isolation methods, as well as establishing new procedures to receive highly pure and morphologically differentiated endothelial, smooth muscle and fibroblastic cells derived from the human penis.

Methods: We evaluated numerous isolation and enrichment techniques using cavernosal tissue from 57 patients. Assessment factors displayed the purity, cell yield, practicability and reproducibility. The purity in cultured cells was analysed using immunocytochemistry and Western blots.

Results: An enzymatic protocol was established for the isolation and cultivation of cavernosal endothelial cells with an impressive purity of 98.0 ± 0.8%. In contrast, already published nearly pure smooth muscle cell cultures were not reproducible in our laboratory. Meaningful evidence for an overwhelming presence of fibroblasts in these widely accepted pure smooth muscle cell cultures is presented.

Conclusion: Endothelial cell cultures derived from human corpora cavernosa are reproducible and reliable to serve for cell culture-based investigations of the endothelial dysfunction. The discrepancy in the purity of smooth muscle cell cultures might reflect laboratory and tissue source factors, lacking an exclusion of fibroblasts in other studies or changes in stromal phenotype under culture conditions. Further research is necessary to clarify a possible plasticity between smooth muscle cells and (myo)fibroblasts and assess functional properties.

Keywords: Andrology; Corpus cavernosum penis; Erectile dysfunction; In vitro models; MACS®; Primary cavernosal cell cultures; Tissue engineering

1. Introduction

During the last decade, the application of various in vitro model systems of isolated corpus cavernosum has significantly enhanced our understanding of the biochemical and physiological mechanisms associated with erectile function [1,2]. Whereas intact corporal tissue consists of a heterogeneous mixture of smooth muscle cells, endothelial cells and fibroblasts [3,4], nearly pure primary cell cultures of the corpus cavernosum in turn offer the possibility to define the

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molecular and functional characteristics of particular cell types [5–15]. In addition to their use in basic research, primary cell cultures can be used for tissue engineering of penile structures [16–18] and will gain increasing importance in the future [18,19]. Interestingly, cell culture-based studies have focused almost exclusively on cavernosal smooth muscle cells. This might be due to the important role these cells play in facilitating erectile function of the penis [9,20] but it disregards the increasing importance of the endothelium and emerging evidence for an association of both erectile and endothelial dysfunction [21].

Despite recent advances, there are still several technical difficulties in obtaining pure primary cell cultures from the corpus cavernosum [6,22]. Although previous studies focused on morphological and functional properties of isolated endothelial or smooth muscle cell cultures, less attention has been paid to detailed isolation protocols, a precise characterisation of the culture composition excluding especially contaminating fibroblasts and a plasticity between smooth muscle cells and (myo)fibroblasts. This sometimes complicated interpretation of the results from cell culture-based experiments [22].

Therefore, we critically studied the available literature dealing with cavernosal cell cultures (about 100 articles) focusing on isolation protocols, the degree of purity and characterization of the cellular phenotype. Subsequent experiments aimed at reproducing reported isolation methods, as well as establishing new procedures for the isolation and cultivation of highly pure and morphologically differentiated endothelial, smooth muscle and fibroblastic cells derived from the human penis.

2. Materials and methods

2.1. Patients

Cavernosal tissue was obtained from potent patients ($n = 57$) aged 17 to 65 years (mean 39; standard deviation ± 13) who were undergoing surgery for either male-to-female transsexual transformation ($n = 33$), penile deviation ($n = 23$) or penile carcinoma ($n = 1$). The study design was approved by the local university ethics committee. Surgical specimens were transported either in ice-cold Dulbecco's Modified Eagle Medium (DMEM) (Biochrom, Berlin, Germany) or Custodiol® (Odyssey Pharmaceuticals, East Hanover, NJ, USA) and immediately used for isolation.

2.2. Isolation of cavernosal endothelial cells

For the enzymatic isolation of endothelial cells cavernosal samples of about 16 mm^3 were used for each isolation assay. These samples were either minced into 1 to 2 mm^3 pieces or left unminced and then incubated in plastic tubes (BD Biosciences, Heidelberg, Germany) containing 5 ml enzyme solution of either collagenase A (0.272 U/mg protein, Roche, Mannheim, Germany) or elastase (3.73 U/mg protein, CellSystems, St. Katharinen, Germany) in a shaker at 37 °C. Enzymes were dissolved in Hanks' balanced salt solution (HBSS) with calcium and magnesium in various concentrations from 0.01% to 0.1% and incubation period ranged from 20 min to 24 hours. Enzymatic digestion was terminated by adding 10 ml DMEM supplemented with 10% fetal calf serum (FCS) (Invitrogen, Karlsruhe, Germany). In some assays the minced tissue pieces were subsequently treated mechanically by pipetting with a 10 ml glass pipette for 2 min. Instead of pipetting, the 16 mm^3 sized complete tissue blocks were repeatedly squeezed with a spatula for 2 min. Afterwards, the suspension was always filtered through a 40 μm nylon mesh to separate single cells from undigested tissue. The filtrate, containing the single cells, was subsequently centrifuged at 200 × g for 10 min, then resuspended and cultivated for 14 days in 75 cm^2 cell culture flasks (Nunc, Wiesbaden, Germany) using 10 ml supplemented endothelial cell growth medium (0.4% endothelial cell growth supplement/heparin, 0.1 ng/ml epidermal growth factor, 1 ng/ml basic fibroblast factor, 1 μg/ml hydrocortisone; PromoCell, Heidelberg, Germany) including 100 U/ml penicillin and 100 μg/ml streptomycin (Biochrom, Berlin, Germany) and 5% FCS.

In addition, some modifications of this standardized protocol were investigated. This included a second re-incubation of incompletely digested tissue and the use of DMEM supplemented with 10% FCS as an alternative culture medium.

2.3. Isolation of cavernosal smooth muscle cells

Explant cell cultures were prepared following the protocols described by other authors [2,3,7,9,11,14,17,23–25]. Briefly, cavernosal tissue was washed in HBSS without calcium and magnesium and cut into 1–2 mm^3 pieces. Segments were placed on 100 mm cell culture dishes (Greiner Bio-One, Frickenhausen, Germany) with a minimal volume of DMEM, supplemented with 20% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin and cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After the explants attached to the substrate, usually within 3 to 4 days, more DMEM containing 10% FCS was added and tissue segments that had detached from the dishes were removed. Cells migrated out from the explants in 4 to 10 days. At this time, the explants were removed, and cells were allowed to achieve confluence.

Moreover, enzymatic cell isolation protocols were applied. As the availability of human cavernosal tissue is limited, we re-incubated undigested tissue after endothelial cell isolation. Different concentrations of collagenase A and elastase from 0.05% to 0.5% were used and the tissue was incubated for 1 to 6 hours to obtain smooth muscle cells. Depending on enzyme concentration and incubation interval, the digestion rate of cavernosal tissue ranged between partial and complete. Untreated fresh tissue was also minced and directly incubated in 0.5% collagenase A for 3 hours in some experiments. Subsequent processing was carried out using the same method employed for the endothelial cells. Finally, cells were cultured for 7 days either in DMEM with 10% FCS and antibiotics or supplemented smooth muscle cell growth medium (PromoCell, Heidelberg, Germany).

2.4. Isolation of tunica albuginea fibroblasts

Tunical fibroblasts were obtained by the explant technique or alternatively enzymatically as previously described for tissue engineering of the tunica albuginea [18]. In the present study these cells only served as internal controls for staining procedures and Western blots.

2.5. Termination of cultivation

The confluency was assessed by means of confocal microscopy. Cells were detached from the culturing surface using 0.05%

trypsin/0.02% EDTA (Biochrom, Berlin, Germany) at 37 °C for 5 min and counted in a hemocytometer. For the characterization of the culture composition various cytospins with 2×10^4 cells/slide were prepared from each culture after the first passage using a Cytospin2® centrifuge (Thermo Shandon, Dreieich, Germany). Selected cultures were additionally seeded on chamber slides (BD Biosciences, Heidelberg, Germany) to study cell morphology (2nd passage). Cultures with the highest cell purity were used in further experiments (2nd and 3rd passage) [8,26].

2.6. Immunocytochemistry

Immunocytochemical staining was performed using the avidin-biotin complex method on cytospins and chamber slides at room temperature. Unless otherwise stated, materials and antibodies were obtained from DakoCytomation, Hamburg, Germany.

After fixing the cells in acetone, quenching of endogenous peroxidase activity and blocking of non-specific binding, slides were incubated with primary antibodies for 1 hour. Whilst the 600+ isolated cultures were screened for the presence of endothelial cells using anti-CD31 antibodies (clone JC70A; 1:300), smooth muscle cell cultures were also stained for desmin (clone DE-R-11; 1:400). As indicated, further characterization of selected isolated cell cultures included staining for prolyl-4-hydroxylase (clone 5B5; 1:100), vimentin (clone V9; 1:200), endothelial specific von Willebrand Factor (clone A0082; 1:800), smooth muscle specific α-actin (clone 1A4; 1:500), smooth muscle specific myosin (Sigma-Aldrich, Munich, Germany; clone hSM-V; 1:500) or fibroblast specific CD90/Thy-1 (dianova, Hamburg, Germany; clone AS02; 1:200). Biotinylated swine anti-rabbit (1:200; for von Willebrand Factor) or horse anti-mouse (1:500; for all other primary antibodies; Vector Laboratories, Burlington, Canada) antibodies were applied for 30 min and followed by incubation with the ABC reagent (Vector Laboratories, Burlington, Canada) for another 30 min. Finally, slides were developed using 3,3′-diaminobenzidine tetrahydrochloride, counterstained with hematoxylin and evaluated in a blinded fashion by counting between 400 and 2000 single cells on each cytospin. Controls included the processing of slides with non-immune IgG instead of primary antibodies, 5 μm sections of frozen human cavernosal tissue and slides with human fibroblasts from the tunica albuginea.

2.7. Western blot analysis

Protein samples were prepared by homogenization of cells in a lysis buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 0.1 mg/ml PMSF, 20 μg/ml aprotinin, and 1 mmol/l Na_3VO_4. Following cell lysis 10 μg protein/slot was electrophoresed in 4–12% NuPAGE® Bis-Tris Gels (Invitrogen, Karlsruhe, Germany) and transferred to polyvinylidene difluoride (PVDF) membranes (Boehringer Mannheim, Mannheim, Germany). After blocking, the samples were incubated at 4 °C overnight incubation with various primary antibodies (CD31: clone JC70A, Dako, Germany, 1:300; eNOS: BD 610296, BD Biosciences, Germany, 1:750; fibroblast specific CD90/Thy-1 clone AS02, dianova, Germany, 1:200). A final incubation was carried out for 2 hours at room temperature with horseradish peroxidase conjugated secondary antibody (NXA 931, Amersham Biosciences, Germany; 1:2000). The reactive bands were detected with a luminol-based kit (ECL, Amersham Biosciences, Germany) using an image analysis system (Image Station Kodak, Germany).

2.8. Magnetic activated cell separation

Magnetic activated cell separation (MACS®) was performed in accordance with the manufacturer's recommendations (Miltenyi Biotec, Bergisch Gladbach, Germany) to removing fibroblasts and enrich the smooth muscle cell cultures. Briefly, 20 μl of Anti-Fibroblast MicroBeads® was added to a heterogeneous cell suspension in 80 μl MACS® buffer (PBS, +0.5% BSA + 2 mM EDTA), before incubated for 30 min at room temperature. Afterwards cells were washed in MACS® buffer, spun, resuspended in 500 μl buffer and applied to an MS Column® in a magnetic separator. While the negative fraction (unlabelled cells) passed through the column, the positive fraction (labelled fibroblasts) was retained and could be elutriated after removal of the column from the magnetic separator.

2.9. Statistical analysis

Statistical differences were determined in SPSS® 11.5.1 for Windows (SPSS®, Munich, Germany) using parametric or non-parametric tests for paired and unpaired samples. When groups were compared, the one way analysis of variance was followed by either Dunnett's T3 or Tukey post hoc multiple comparison analysis. Results are expressed as mean ± standard deviation; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

3. Results

3.1. Isolation and characterization of cavernosal endothelial cells

About 70 different modifications of enzymatic isolation techniques for cavernosal endothelial cells were investigated in our study to finally obtain highly pure endothelial cell cultures. Only those procedures with a promising outcome of specific cell retrieval were further optimized and reproduced in a large number of cases, providing excellent reproducibility in tissues from different patients.

Cavernosal endothelial cells cultured in tissue flasks demonstrated typical cobblestone morphology and contact inhibition [16,27,28] with maximal densities of 1000 cells/mm^2. Endothelial cell viability and differentiation [8,26] could be maintained for months when cultured in endothelial cell specific culture medium. On the other hand, the majority of endothelial cells (>95%) did not survive cultivation periods longer than 14 days when DMEM supplemented with 10% FCS was used.

The exposure of the previously digested tissue to mechanical force [29] by pipetting with a glass pipette resulted in a 12.5× increase in cell quantity ($p < 0.01$) and an increased endothelial purity ($p < 0.01$) in the isolated cultures. It was therefore applied in all final experiments.

Cell isolation with collagenase A followed by a cultivation period of 14 days exhibited an unfavourable increase in contamination by stromal cells with rising enzyme concentrations from 0.02% to 0.1% at identical incubation periods ($p < 0.05$). This effect was less pronounced in cultures isolated with elastase and was

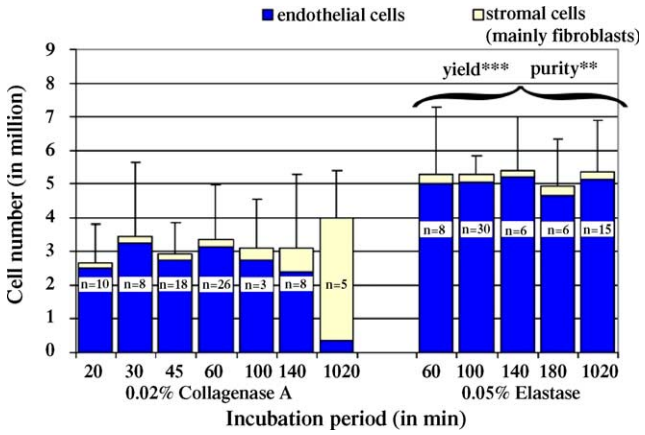

Fig. 1. Enzymatic isolation of endothelial cells with either 0.02% collagenase A or 0.05% elastase and cultivation for 14 days. Improved cell yield and purity with elastase (***$p < 0.001$ and **$p < 0.05$ respectively) independent from duration of enzyme incubation. (Cell count is expressed as mean ± standard deviation; the number of processed samples for each procedure is illustrated.)

virtually eliminated by using overnight incubations with elastase in concentrations between 0.01% and 0.05%. While the use of 0.02% collagenase A resulted in a significant increase in non-endothelial cells with longer incubations ($p < 0.01$), the culture contamination was consistently lower for varying incubation periods with 0.05% elastase (Fig. 1) ($p < 0.05$). Moreover the final yield of endothelial cells was significantly improved after the cultivation period of 14 days with 0.05% elastase when compared to 0.02% collagenase A ($p < 0.001$) (Fig. 1).

Finally the incubation of intact tissue blocks instead of minced tissue pieces with elastase and additional squeezing of these tissues with a metal spatula for 2 min revealed a further increase in the degree of purity to 98.0 ± 0.8% ($p < 0.001$). As the extraction of endothelial cells from these tissues blocks appeared to be nearly complete in histological controls (Fig. 2), this isolation procedure was considered to be the optimum in our study. In 18 experiments with cavernosal specimens from 13 different patients, on average 98.5% of the cells in these cultures expressed CD31 and 97.5% von Willebrand Factor, while only 1.8% stained positive for a fibroblast specific marker and less than 0.05% for desmin after the first passage (Fig. 3). Western blotting confirmed these endothelial characteristics showing single bands of 130 kDa for CD31 and 140 kDa for eNOS, whilst lacking 30 kDa fibroblast marker bands which were strongly expressed in tunical fibroblast (Fig. 4).

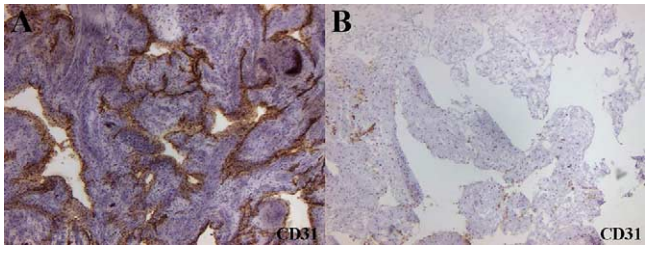

Fig. 2. Immunohistochemical detection of the endothelial marker CD31 in 5 μm sections of human corpus cavernosum (original 40× magnification). (A) In native specimens endothelial cells are completely lining the sinus. (B) After enzymatic isolation of endothelial cells with 0.05% elastase for 100 min only a few remnants of the endothelial layer are detectable.

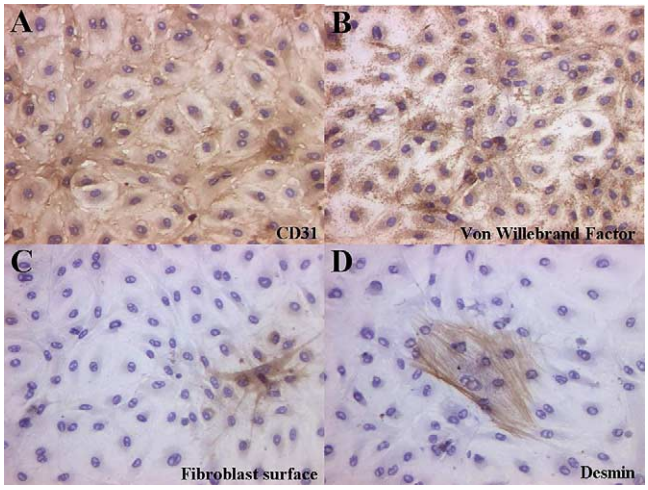

Fig. 3. Enzymatically isolated endothelial cell culture from human corpus cavernosum (original 200× magnification). (A) 98.5% cells stained positive for CD31 (endothelial marker). (B) 97.5% cells expressed von Willebrand Factor (endothelial marker). (C) 1.8% fibroblasts contaminated the cultures (ASO2 fibroblast marker). (D) Only 0.05% desmin positive cells (smooth muscle cells).

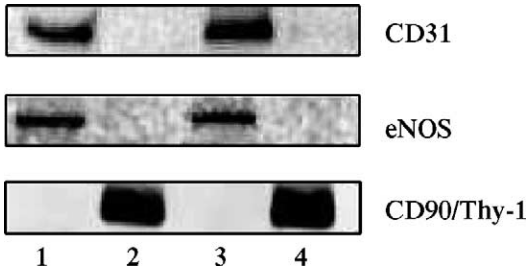

Fig. 4. Western blot analysis of endothelial cell specific CD31 expression, eNOS protein and fibroblast specific CD90/Thy-1 expression in penile cell cultures. Lanes 1 and 3 correspond to endothelial cells isolated from two different transsexual patients soaked in 0.05% elastase overnight and squeezing the 16 mm^3 tissues with a metal spatula subsequently. Lanes 2 and 4 are the result of tunical fibroblasts isolated using the explant technique from two further transsexual patients. These findings confirm the purity of the endothelial cell cultures in lanes 1 and 3, which demonstrate single bands of 130 kDa for CD31 and 140 kDa for eNOS, but lack the 30 kDa fibroblast marker bands which are strongly expressed in tunical fibroblast (lanes 2 and 4).

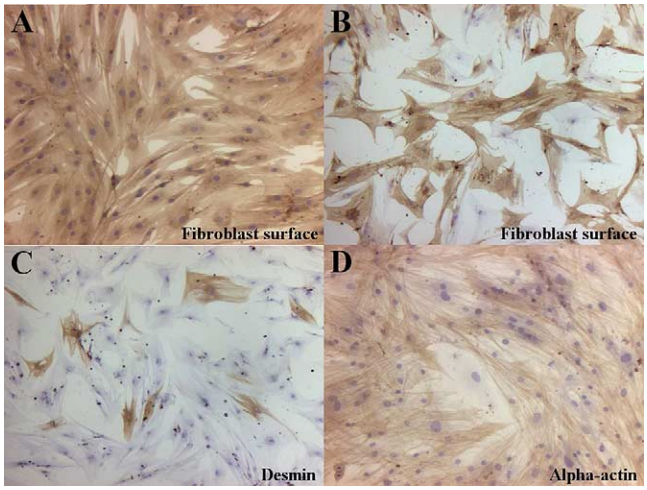

Fig. 5. Stromal cell cultures isolated from human corpus cavernosum by explant technique. (A) More than 95% of the cells that migrated out of explants expressed the fibroblast marker AS02 (original 100×). (B) Staining for fibroblasts (original 40×) and (C) desmin (original 40×) demonstrated no difference in cell morphology of smooth muscle cells and fibroblasts. (D) α-actin staining in cell multilayers pretended the existence of homogenous smooth muscle cell cultures, but when seeded in lower densities similar results to (C) were obtained (original 100×).

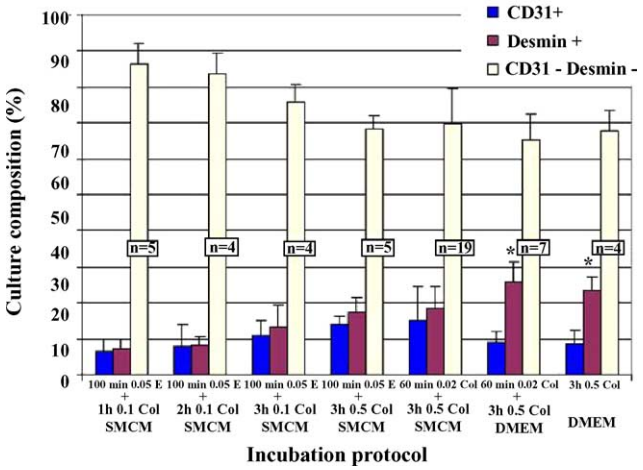

Fig. 6. Relative culture compositions for different isolation protocols using elastase (E) and/or collagenase (Col) as enzymes, and Dulbecco's Modified Eagle Medium (DMEM) or a specific smooth muscle cell medium (SMCM) as cell medium. After the usual cultivation period of 7 days, immunocytochemical staining revealed endothelial cells (CD31 +), smooth muscle cells (desmin +) and fibroblasts (CD31 - and desmin −). A partial digestion of the cavernosal tissue resulted in a low cell yield of smooth muscle cells (desmin +) (columns 1–3), whilst a complete digestion was necessary to obtain higher percentages of smooth muscle cells (desmin +) (columns 4 and 5). This could be augmented by using DMEM ($*p < 0.05$; columns 6 and 7) instead of a specific smooth muscle cell medium (columns 4 and 5) due to a slower proliferation of desmin negative stromal cells (e.g. fibroblasts) and unfavourable conditions for endothelial cells. (Culture composition is expressed as mean ± standard deviation; the number of processed samples for each procedure is illustrated.).

3.2. Isolation and characterization of cavernosal smooth muscle cells

Morphologically homogeneous explant cell cultures [11,12,24,25] were obtained from 20 different potent patients. Cultivated either in DMEM or in a specific smooth muscle cell medium, cells migrated out from the tissues after 4–10 days and confluence was achieved after 3–4 weeks. When these primary cultures were transferred on cytospins for immunocytological characterization they revealed a very low positive staining for smooth muscle cell markers (desmin, α-actin, myosin; in all cases <5%), a nearly complete absence of endothelial cells (<1%), but highly expressed fibroblastic cell markers (>95%) (Fig. 5A).

In enzymatically isolated cultures larger quantities of differentiated smooth muscle cells were obtained. When immunostained for cell specific markers the desmin/myosin and desmin/α-actin ratios were 1:1.14 ($n = 30$) and 1:1.85 ($n = 32$; $p < 0.001$) respectively [2]. In these cultures smooth muscle cells and fibroblasts were morphologically indistinguishable (Fig. 5B and C). Staining for smooth muscle cell markers in confluent and multilayered cell cultures [17] pretended homogeneity of smooth muscle cells (Fig. 5D), but when the same cultures were seeded in a lower density many unstained cells were detectable.

For the enzymatic isolation, specimens were pre-incubated using either 0.05% elastase for 100 min or 0.02% collagenase for 60 min to harvest endothelial cells. Then, in a second step, collagenase A in a concentration of 0.5% completely dissociated the cavernosal tissue within 3 h. This procedure ($n = 24$) resulted in a high total cell yield of about 11×10^6 cells after an additional cultivation period of 7 days. An average of 18.3 ± 5.7% of these cells expressed desmin, which was the highest smooth muscle cell content in this series (Fig. 6, column 4 and 5). An augmentation to a mean value of 25.8 ± 5.6% of smooth muscle cells ($p < 0.05$) was achieved by utilising DMEM instead of smooth muscle cell medium due to a slower proliferation of desmin negative stromal cells (e.g. fibroblasts) and unfavourable conditions for endothelial cells ($n = 7$) (Fig. 6, column 6). Interestingly, comparable outcomes were obtained when pre-incubation for endothelial cell isolation was omitted and 0.5% collagenase A was directly applied for 3 h followed by cultivation in DMEM (Fig. 6, column 7).

Modified incubation periods with less concentrated enzymes (Fig. 6, columns 1 to 3), multiple incubation steps for a fractional isolation [11] or pipetting of undigested tissue [10,11,13,15] (data not shown) did not improve cell yield or percentages of desmin positive cells.

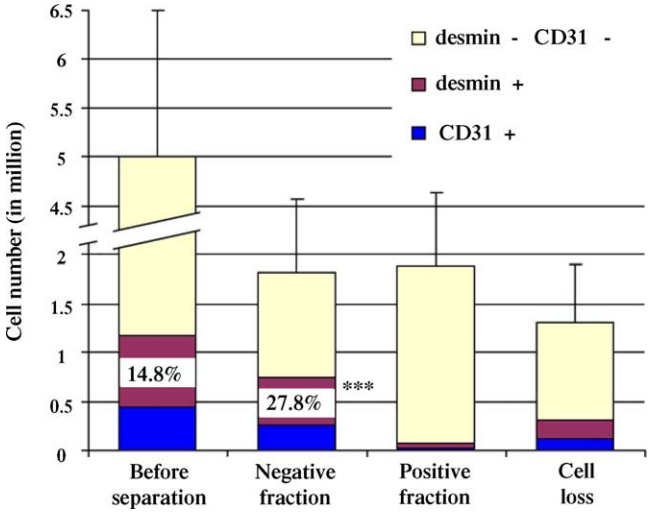

Fig. 7. Magnetic activated cell sorting (MACS®) using beads against fibroblasts to transiently retain labelled cells in MS Columns® (positive fraction), while unlabelled cells passed through the columns (negative fraction). The immunocytochemical staining revealed endothelial cells (CD31 +), smooth muscle cells (desmin +) and fibroblasts (CD31 - and desmin −). Percentages of smooth muscle cells (desmin +) significantly increased from 14.8 before the separation to 27.8 (***$p < 0.001$) ($n = 11$; percentages are expressed as mean; cell count is expressed as mean ± standard deviation).

3.3. Magnetic cell separation

Magnetic cell separation with a fibroblast specific antibody enabled further purification of heterogeneous cultures. The percentage of smooth muscle cells (desmin positive) nearly doubled from 14.8 ± 6.7% before magnetic separation to 27.8 ± 7.2% in the negative fraction directly afterwards ($n = 11$; $p < 0.001$). On average in the positive fractions about 1% of cells expressed endothelial markers ($n = 11$; $p < 0.001$) and nearly 3% expressed smooth muscle cell markers ($n = 11$; $p < 0.001$), while >95% exhibited positive staining for fibroblast specific proteins and enzymes (Fig. 7). Therefore, the positive fractions were considered to consist mainly of cavernosal fibroblasts (>95%) and were utilized in further experiments [26].

4. Discussion

Intact corporal tissue involves a balance of different cell types including mainly endothelial cells, smooth muscle cells and fibroblasts to provide altogether an optimal erectile function [3,4]. Although the properties of a particular cell type might be best characterised when interacting with other cell types, extracellular matrix or blood (in vivo [17], organ bath [3,5,6,15], co-culture [16]), it is just the main advantage of nearly pure primary cell cultures to study one specific cell type [27]. However, impurity and dedifferentiation of isolated cell cultures complicate the interpretation of results from cell culture-based experiments [22]. Therefore the aim of the present study was to improve our knowledge of cell isolation techniques from the human corpus cavernosum. We did not perform experiments with non-human tissue and therefore can not comment on whether the presented results might be valid for other species.

The isolation and cultivation of a specific cell type required different protocols to achieve a high degree of viability and purity for each cell type. Smooth muscle cells and fibroblasts are strongly embedded in an extracellular matrix formed mainly of collagen. The endothelial cell layer covers this stromal tissue and forms the vascular spaces [4]. Therefore the endothelium has the first contact with the enzyme solution and single endothelial cells can be harvested at a time when the stromal tissue is nearly undigested. In contrast large numbers of stromal cells can only be isolated by almost completely digesting the extracellular matrix with collagenase.

In a large series of 57 human tissue specimens we established a simple, but very effective new protocol for the enzymatic isolation of cavernosal endothelial cells based on the basic research of many other investigators. The high degree of purity (98.0 ± 0.8%) in these primary endothelial cultures is unique. This is most likely because, in contrast to collagenase [5,16,28,29], it is impossible to digest the extracellular matrix of the stromal cells using elastase. Therefore the enzymatic digestion with elastase combined with a subsequent mechanical force [29] mainly supports the release of endothelial cells from the basement membrane, but leaves the stromal cells in their extracellular collagen matrix almost untouched. Because the contamination by fibroblasts is reduced to a minimum of 1.8%, our protocol seems to be favourable over other protocols described in the literature involving complicated purification procedures like cloning cylinders, magnetic beads, fluorescence activated cell sorting (FACS) or the use of gelatine coated culture flasks [5,16,27,28]. Our enzymatic procedure provided a high cell yield immediately, even from small biopsies [8,26]. This is clearly an advantage over time-consuming explant techniques requiring a high population doubling rate to achieve a comparable cell yield from limited biopsy material [27]. Finally, enzymatically isolated endothelial cells retained their phenotypic characteristics in our cell cultures including cobblestone morphology, contact inhibition and expression of cell specific proteins (CD31, von Willebrand Factor and eNOS), confirming similar findings [16,28]. Although mainly morphological criteria are provided

in this study to assess the phenotype, functional properties in subsequent investigations [8,26] underline the phenotype being well conserved in vivo.

Because the change of cavernosal smooth muscle tone has emerged as the key mechanism in erectile function [20], numerous studies have focused on cell culture models of penile smooth muscle cells since their introduction in 1988 [7]. In the past smooth muscle cell character and the identity of the isolated and investigated cavernosal stromal cell cultures was rarely doubted, but Granchi et al. recently demonstrated the apparent difficulties in obtaining pure and differentiated cultures of human adult cavernosal smooth muscle cells without contamination by fibroblasts [6]. They consequently preferred the term 'stromal cells' and utilized human fetal penile cells in further experiments, that were easier to isolate and showed a more favourable rate of differentiated smooth muscle cells [6]. Rees et al. also discussed the difficulties in harvesting pure smooth muscle cell cultures using the cavernosal explant technique [14] and Zheng et al. established a phenotypically stable smooth muscle cell line from the adult human corpus cavernosum by enzymatic cell isolation and subsequent subculture of small islands of <10 cells [22].

Numerous studies described morphologically homogeneous cavernosal cell cultures [7,12,17,24,25] thought to consist almost entirely of smooth muscle cells on the basis of mainly morphological descriptions examined by light microscopy. However we observed smooth muscle cells and fibroblasts in such cultures using immunocytochemical staining. Our results illustrate that microscopic distinction of spindle-shaped smooth muscle cells and very flattened and spread out shaped fibroblasts [11,24] is not supportable in human cavernosal cell cultures. In agreement with earlier work [7,30] smooth muscle cells and fibroblasts demonstrated similar morphological features. This underlines the importance of performing immunocytochemistry to further characterize isolated stromal cells. Numerous authors demonstrated the ''cellular homogeneity'' of their cultures by the presence of positive smooth muscle specific immunoreactivity for either α-actin, myosin or desmin [9,11,12]. But this concept might be misleading because most of these studies lacked counterstainings with non smooth muscle cell markers or additional fibroblast cultures as negative controls. Interestingly, only a minority of authors specified exact percentages of positively stained cells [3,6,31], whilst others even omitted a characterization of the culture composition completely [10,15]. It might be redundant to rule out the presence of endothelial cells by means of immunocytochemical studies, as has been suggested before [6,23,25], because endothelial cell contamination is easily detectable by cell morphology. In addition, we could support the findings that endothelial cells are not able to survive cultivation in DMEM over more then one passage [27,32]. The main focus of the isolated cell cultures should have been the exclusion of fibroblasts, but none of the more than 100 published studies has ever involved staining with anti-fibroblast antibodies, although these have been available for human and other species for some years [33]. This is perhaps for a good reason: staining with specific anti-fibroblast-antibodies in our ''smooth muscle cell cultures'' harvested using the explant technique revealed more than 95% immunopositive cells.

In contrast to most studies [2,7,11,12,24,25], we investigated the isolation of smooth muscle cells from a large number of patients ($n = 57$). It is well known that the availability of normal functional tissue is a problem for all studies in this cell culture work. However, we deliberately did not use cavernosal tissue from penile implant surgery, as utilized by others [2,11,12,17,23–25], because of the reduction in smooth muscle content demonstrated in these specimens [34–36]. Nevertheless, we could not reproduce the above mentioned results of others, although tissue samples were derived mainly from male-to-female transsexual transformation ($n = 33$) and penile deviation surgery ($n = 23$). Explant cell cultures in our study revealed a very low positive staining for smooth muscle cell markers ($<5\%$ in all cases) and were clearly inferior to enzymatically isolated cultures, which had a maximum of about 25% smooth muscle positive cells.

Considering immunocytochemical data, desmin proved to be the most reliable marker for smooth muscle cells, because it was only rarely detectable in fibroblast cultures purified using fibroblast specific magnetic beads. Whereas especially α-actin was also sometimes expressed in these fibroblastic cultures. The latter aspect might involve the phenomenon of fibroblast to myofibroblast transition [37].

Stromal cells in our cultures (only 1st and 2nd passage) demonstrated varying degrees of α-actin expression, as described by Campos de Carvalho et al. [24]. This could be related to the well known decrease in myofilaments when cultured smooth muscle cells change from the contractile phenotype to a synthetic phenotype involving multiple passages [22,38]. This aspect appears to be particularly important as some ''fibroblast markers'' detecting for example prolyl-4-hydroxylase might demonstrate a false positive signal in the synthetic phenotype of smooth muscle cells. Nevertheless, it has to be considered, that

the percentage decrease in smooth muscle cell markers in higher passages might be related to an increase in fibroblasts due to their extensive proliferative capacity [33]. Despite the smooth muscle cell plasticity, it has been demonstrated, that many in vivo features of corporal cell physiology and pharmacology are preserved in short term cell cultures, and further research under carefully defined experimental cell culture conditions seems to be justified [7,14,16,17,22,23,28].

Our findings of about 25% desmin-positive smooth muscle cells with a cultivation period of 7 days after cell isolation are in agreement with the recent study by Malysz et al. [13], who isolated cells enzymatically from rabbit corpus cavernosum and reported 20–30% myocytes based on an immediate staining of the isolated cells with anti-α-actin and anti-myosin antibodies. These results contradict publications negating the existence of fibroblasts – without presenting any excluding proofs – in stromal cell cultures derived from the human corpus cavernosum [12,24]. Our investigations clearly cast some doubt on the stated purity of cavernosal smooth muscle cell cultures utilized in other studies. This aspect appears to be particularly important as it might raise questions about the value of studies using heavily fibroblast contaminated smooth muscle cell cultures.

5. Conclusions

We established a simple but highly effective protocol for the isolation and cultivation of endothelial cells derived from the human corpus cavernosum as an optimal precondition for the further cell culture-based investigation of endothelial function in the erectile process.

We have also presented meaningful evidence of an overwhelming presence of fibroblasts in widely accepted pure smooth muscle cell cultures from human cavernosal tissue. Further research is necessary to fully characterize the phenotype of such cavernosal stromal cell cultures in terms of morphological and functional properties and clarify a possible plasticity between smooth muscle cells and (myo-)fibroblasts. This will have a bearing on both basic research and tissue engineering of penile structures in the future.

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Editorial Comment

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Erectile dysfunction (ED) is related to ‘‘endothelial dysfunction’’ and may emerge as ‘‘the tip of the iceberg’’ of systemic vascular disorder. To clarify ED biological basis, it should be also focused on isolated endothelial and stromal cells. Although i*n vitro* cell cultures have been rising up as an useful tool to study ED, culture purity is the limit to deal with, as for any *in vitro* model. Pilatz et al. pointed out these practical boundaries and purpose an innovative technique to purify endothelial and smooth muscle cells from human adult corpora cavernosa. Many of cell culture-based studies, limited to penile stroma cells, refer to ‘‘smooth muscle cells’’, disregarding more specific characterization. First, the endothelial cell culture purity (98%), shown by Pilatz et al., is quite impressive and simply obtained, avoiding time-consuming procedures. Second, as observed by Granchi et al. [1], it is not easy to obtain pure adult penile smooth muscle cells, without fibroblast contamination. They, indeed, overcame this problem using for the first time human fetal penile smooth muscle cells, extensively characterized and successfully utilized in basic research [2]. Cells from fetal tissues allow a negligible fibroblast contamination as compared to adult ones. Managing ‘‘pure’’ cultured cells is, indeed, a very tricky deal, because of *in vitro* plasticity in smooth muscle cell/myofibroblast/fibroblast phenotype. The distinction of smooth muscle cells from fibroblast and the switch from the contractile to mitotic phenotype in primary culture have been described since quite long ago. Different phenotypes reflect different metabolic cell state, associated to specific function, such as migration, proliferation or extracellular matrix deposition. Thus, the *in vitro* cell system purity is essential to avoid any misleading in result interpretation. In medical science, basic research is often linked to clinical application, therefore achieving ‘‘pure’’ cell cultures seems likely the unique tool to correctly approach cell-based research. The ultimate goal of this basic research, along with clinical efforts, is to provide a rational scientific basis for *in vitro* tissue engineering and patient-specific therapies.

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