Marker profile for the evaluation of human umbilical artery smooth muscle cell quality obtained by different isolation and culture methods

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Abstract Even though umbilical cord arteries are a common source of vascular smooth muscle cells, the lack of reliable marker profiles have not facilitated the isolation of human umbilical artery smooth muscle cells (HUASMC). For accurate characterization of HUASMC and cells in their environment, the expression of smooth muscle and mesenchymal markers was analyzed in umbilical cord tissue sections. The resulting marker profile was then used to evaluate the quality of HUASMC isolation and culture methods. HUASMC and perivascular-Wharton's jelly stromal cells (pv-WJSC) showed positive staining for α -smooth muscle actin (α -SMA), smooth muscle myosin heavy chain (SM-MHC), desmin, vimentin and CD90. Anti-CD10 stained only pv-WJSC. Consequently, HUASMC could be characterized as α -SMA⁺, SM-MHC⁺, CD10[−] cells, which are additionally negative for endothelial markers (CD31 and CD34). Enzymatic isolation provided primary

HUASMC batches with 90–99 % purity, yet, under standard culture conditions, contaminant CD10⁺ cells rapidly constituted more than 80 % of the total cell population. Contamination was mainly due to the poor adhesion of HUASMC to cell culture plates, regardless of the different protein coatings (fibronectin, collagen I or gelatin). HUASMC showed strong attachment and long-term viability only in 3D matrices. The explant isolation method achieved cultures with only 13–40 % purity with considerable contamination by CD10⁺ cells. CD10⁺ cells showed spindle-like morphology and up-regulated expression of α -SMA and SM-MHC upon culture in smooth muscle differentiation medium. Considering the high contamination risk of HUASMC cultures by CD10⁺ neighboring cells and their phenotypic similarities, precise characterization is mandatory to avoid misleading results.

Keywords Human umbilical artery smooth muscle cells (HUASMC) · Wharton's jelly stromal cells · Isolation · Characterization · Markers · CD10

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Introduction

Vascular smooth muscle cells (VSMC) constitute the internal layer of the blood vessels, the tunica media. Together with endothelial cells, VSMC are responsible for the regulation of the blood flow and the immunomodulation of inflammatory processes in the

vascular wall and the surrounding tissue. The mechanical and immunomodulatory functions of these cells are strictly connected and important for the maintenance of healthy blood vessels. The processes regulating VSMC phenotype changes, proliferation, migration, apoptosis and ECM synthesis play key roles in pathologies such as atherosclerosis (Orr et al. 2010). Cell culture VSMC models are needed since in vitro experiments represent the first necessary step in vascular biology research.

The human umbilical cord arteries represent an easy and low-cost source of VSMC, since alternative access to human vascular tissue is limited to material obtained by surgical operation, but their isolation is hindered by the lack of a complete marker profile.

Various contractile, cytoskeletal and cytoskeletal-bound proteins have been used to confirm the smooth muscle origin of the cells, e.g. α -smooth muscle actin (α -SMA), smooth muscle myosin heavy chain (SM-MHC), desmin, smoothelin, h-caldesmon, h-calponin, SM-22 α and metavinculin. The use of these markers, however, has some limitations. The majority of these proteins are expressed especially when VSMC show a quiescent-contractile phenotype. When activated in vivo or cultured in vitro, VSMC switch to a proliferating-secretive phenotype and lose the expression of many contractile proteins (α -SMA, SM-MHC, h-caldesmon and smoothelin; Owens et al. 2004). Moreover, other markers (α -SMA, SM-22 α , h-calponin and metavinculin) are also expressed in different cell types, including stromal cells and activated myofibroblasts (Sartore et al. 2001). To date, a specific vascular smooth muscle marker detecting both quiescent and proliferating cells is still unknown.

This consideration is particularly important when dealing with HUASMC, since contamination by neighboring Wharton's jelly stromal cells is facilitated by the lack of well-defined borders between the vascular smooth muscle and the perivascular mucous tissue of Wharton's jelly. Cells from perivascular Wharton's jelly, further referred to as perivascular Wharton's jelly stromal cells (pv-WJSC), show mesenchymal stem cell characteristics, including the expression of CD10, CD44, CD73, CD90 and CD105, and can be differentiated into chondrocytes, osteocytes and adipocytes (La Rocca et al. 2009). Additionally, these cells express typical VSMC markers, such as α -SMA, desmin and h-caldesmon (Farias et al. 2011; Corrao et al. 2013).

In many previous studies, characterization of primary HUASMC has been limited to the expression of α -SMA (Olafsson et al. 2012; Rainger et al. 2001; Martin de Llano et al. 2007). Although other groups also included negative staining for endothelial and fibroblast markers (Cairrao et al. 2009), no standard marker profile has yet been established.

The aim of this study was to provide an overview of the expression of VSMC and mesenchymal markers in umbilical cord tissue, which is helpful for the characterization of HUASMC after isolation and during culture. Based on the studies of Nanaev et al. (1997) and Farias et al. (2011), we further analyzed the expression of SM-MHC, which is considered to be the most specific marker of contractile VSMC, and the expression of the common fibroblast and mesenchymal marker CD90. As two main approaches have been suggested for HUASMC isolation, based on the outgrowth of cells from tissue explants (Rainger et al. 2001; Martin de Llano et al. 2007) and on tissue enzymatic digestion (Cairrao et al. 2009), we intended to assess the purity outcome of the two isolation methods by immunochemical analysis.

Cells cultured on different 2D substrates were characterized to quantify the purity loss in vitro. Further, the viability, spreading and cell–cell interactions of HUASMC cultured in 3D matrices were analyzed. Finally, it was tested if a characterization based on the expression of contractile proteins could be sufficient to segregate HUASMC and pv-WJSC in vitro.

Materials and methods

Characterization of umbilical cord tissue and isolation of HUASMC

Umbilical cords were collected after normal-term pregnancies following the principles of the Declaration of Helsinki with informed consent. Cryosections (5 μ m, $n = 3$) of umbilical cord samples were mounted on microslides (Assistant, Karl Hecht AG, Sondheim, Germany), air-dried overnight, and stored frozen. Prior to immunostaining, tissue sections were fixed in acetone for 4 min. Slides were immunolabeled using the ultravision LP detection system (Thermo Scientific, Fremont, CA, USA) according to the manufacturer's instructions. The following antibodies

were diluted in antibody diluent (Dako, Glostrup, Denmark) and applied for 30 min at room temperature: CD34 (0.5 µg/mL, Dako), CD10 (0.8 µg/mL, Dako), desmin (0.1 µg/mL, Dako), vimentin (0.16 µg/mL, Dako), CD90 (0.06 µg/mL, BD Biosciences, San Jose, CA, USA), SM-MHC (0.65 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) and α -SMA (0.3 µg/mL, Sigma-Aldrich). IgG controls (Ancell, Bayport, MN, USA) were used at the same concentrations as the respective antibodies. After three washing steps in PBS, slides were incubated with primary antibody enhancer for 10 min, followed by HRP-polymer for 15 min. The slides were washed again three times in PBS, and immunolabeling was visualized by a 5-min exposure to 3-amino-9-ethylcarbazole (AEC, all from UltraVision kit, Thermo Scientific). The slides were counterstained with Mayer's hemalum (Merck, Darmstadt, Germany), washed in distilled water and mounted with Kaiser's glycerol gelatin (Merck).

To isolate HUASMC by enzymatic digestion, the Wharton's jelly was removed and the isolated artery pieces (minimum 6-cm long) were kept in ice cold HBSS (Sigma-Aldrich) combined with penicillin–streptomycin (Sigma-Aldrich). After dissection, the arteries were cut into 2-cm long pieces and digested in dispase (50 CU/mL)(BD Biosciences) for 10 min at 37 °C. After digestion, the Wharton's jelly could be completely removed from the medium. The endothelium was scrubbed away by using a sterile gauze bead on a cannula. The pieces were further cut and digested in MOPS buffer (Sigma-Aldrich) containing crude collagenase Type II from *Clostridium histolyticum* (2 mg/mL; Sigma-Aldrich) and 5 mM CaCl_2 at 37 °C under gently swirling. After 1 h, the tissue was almost completely digested and SMC growth medium was added 1:1 to the cell suspension, which was filtered through a 70-µm cell strainer (BD Biosciences). Cells were centrifuged at 650 g for 15 min without break, washed in PBS and plated in 6 well plates (Cellstar® Greiner bio-one, Frickenhausen, Germany) at a density of 1.4×10^5 cells/well in SMC growth medium (Promocell, Heidelberg, Germany) containing 5 % FBS, epidermal growth factor (0.5 ng/mL), basic fibroblast growth factor (2 ng/mL), insulin (5 µg/mL), HEPES (20 mM, Sigma-Aldrich) and gentamicin–amphotericin (Life Technologies, Carlsbad, CA, USA). The cells were counted (TC10, BioRad, Hercules, CA, USA) and plated with an average cell viability of 95 %. Freshly isolated cells were

characterized by flow cytometry (2×10^5 cells/sample) and western blotting (3×10^5 cells/sample).

Alternatively, to isolate the cells by the explant method (Martin de Llano et al. 2007), the lumen of the arteries was first perfused with HBSS and then completely filled with dispase (50 CU/mL, BD Biosciences). The edges of the arteries were clamped and the vessel was incubated at 37 °C for 10 min. Afterwards, the arteries were perfused with HBSS to collect the endothelial cells. After perfusion and endothelial removal, the arteries were cut in pieces (approximately 3-mm long). The tissue pieces were evenly distributed into a well of a six well plate (Cellstar 211®, Greiner bio-one) with the lumen facing the bottom (five pieces/well). SMC growth medium (Promocell, Heidelberg, Germany) containing 5 % FBS, epidermal growth factor (0.5 ng/mL), basic fibroblast growth factor (2 ng/mL), insulin (5 µg/mL), HEPES (20 mM, Sigma-Aldrich) and gentamicin–amphotericin (Life Technologies) was added dropwise to avoid detaching of the explants. After 1 week the cells started to outgrow from the explants and to form colonies. Three weeks after explant dissemination cells reached a cell confluence of 50 % with highly dense cell areas close to the explant (P0). To avoid detachment of cells in the highly confluent regions, cells were passaged before reaching confluence. Further passaging of cells was done at 80–90 % confluence by splitting the cell population in a ratio of 1:3 (approximately after 7 days). Cells were harvested for analysis in P0, P1 and P2 with Accutase® (Sigma-Aldrich).

Effectiveness of both methods was evaluated at the same time point in the culture of the cell populations. Quality and purity of isolated cells from explant and enzymatic digestion method were compared at 80–90 % confluence 4 weeks post isolation (one passage after 3 weeks).

2D culture of HUASMC by enzymatic digestion

Freshly isolated cells were seeded in SMC growth medium (Promocell, Heidelberg, Germany) containing 5 % FBS, epidermal growth factor (0.5 ng/mL), basic fibroblast growth factor (2 ng/mL), insulin (5 µg/mL), HEPES (20 mM, Sigma-Aldrich) and gentamicin–amphotericin (Life Technologies) in six wells plates or T25 flasks (Cellstar 243® Greiner bio-one) treated with different protein coatings, which

were tested to optimize cell adhesion and maintain population purity. Briefly, coating of plates with fibronectin (Sigma-Aldrich) was done at a density of $5 \mu\text{g}/\text{cm}^2$; coated plates were air-dried at RT overnight. Coating with collagen type I ($10 \mu\text{g}/\text{cm}^2$, Sigma-Aldrich) was done following the supplier's instructions; before use, plates were sterilized with UV. Coating with gelatin was done using a sterile 0.1 % gelatin solution (ICN Biomedicals Inc., Santa Ana, CA), $2 \text{ mL}/10 \text{ cm}^2$ tissue culture surface at 37°C overnight. Cells reached an 80–90 % cell confluence after 3 weeks (P0). Further passaging of cells was done after cells reached 80–90 % confluence by splitting the cell population in a ratio of 1:3 (approximately after 7 days). Flow cytometry was done after P0 and P1 in order to confirm the purity of the population.

3D culture of HUASMC by enzymatic digestion and calcein AM live staining

After isolation, cells were embedded in Matrigel™ (BD Biosciences, cat. 354234; 10^6 cells/mL) in 96-well black microplates with μClear bottom (Greiner bio-one) and cultured in SMC growth medium. During culture, the cell arrangement was analyzed by live staining with calcein AM. Gels were washed with sterile PBS and incubated in $2 \mu\text{M}$ calcein AM solution (Life Technologies) for 30 min at 37°C . Analysis was done using a TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany).

In preliminary experiments, cells were embedded in collagen type I gels (rat tail, $6 \text{ mg}/\text{mL}$, BD Biosciences) as well; but the method was soon abandoned due to fast gel compaction, which impaired cell visualization.

Spindled index

The qualitative spindled index was used to classify the formation of lamellipodia extensions of HUASMC within the 3D Matrigel. As published by Dikovskiy et al. (2008) the spindled index was ranked based on the appearance of the cellular lamellipodia: 4, highly spindled with regular lamellipodia; 3, spindled with frayed lamellipodia; 2, nonspindled with frayed lamellipodia; 1, rounded with minor lamellipodia; 0, completely rounded with no lamellipodia.

2D culture of HUASMC by explant method and immunofluorescence characterization

Expression of CD10 and CD31 was analyzed by flow cytometry in P0, P1 and P2. Cells in P2 were cultured for 6 days in Medium 231 supplemented with Smooth Muscle Differentiation Supplements (SMDS; Life Technologies) giving a final concentration of 1 % FBS and $30 \mu\text{g}/\text{mL}$ heparin. Immunofluorescence staining of $\alpha\text{-SMA}$ and SM-MHC was performed before and after incubation in differentiating medium. Cell layers were washed with PBS, fixed and permeabilized (BD Cytotfix/Cytoperm). After blocking in 5 % goat serum, cells were incubated overnight at 37°C with primary anti- $\alpha\text{-SMA}$ and anti-SM-MHC antibodies (same antibodies as for western blotting) diluted 1:50. Secondary goat anti-mouse FITC-conjugated antibody (Jackson ImmunoResearch, West Grove, PA, USA) was incubated for 1 h at RT. Images were taken with an up-right Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan).

Flow cytometry

After detachment of the cells with Accutase® (Sigma-Aldrich), the cells were washed once with ice-cold PBS and centrifuged at 230 g for 7 min at 4°C . The cell pellet was resuspended with ice-cold PBS containing 2 % FBS and incubated with specific antibodies for 30 min at 4°C . Cells were stained with monoclonal antibodies against CD10 (PE-labeled, clone HI10a), CD31 (FITC-labeled, clone WM59) or isotype controls IgG $_{1\kappa}$ FITC- or PE-labeled (all purchased from BD Biosciences) and analyzed using a FC500 cytometer (Beckman-Coulter, Brea, CA, USA). Freshly isolated cell suspensions obtained by enzymatic digestion were further stained with CD29 (APC labeled, clone MAR4; integrin $\beta 1$) to exclude blood cells from the analysis. CD10 (CALLA) and CD31 (PECAM) were used to identify mesenchymal stromal cells derived from the perivascular Wharton's jelly and endothelial cells, respectively. CD10 and CD31 immunonegative cells were characterized as HUASMC. Data analysis was done with FlowJo (Tree Star Inc., Ashland, OR, USA).

Western blotting

Cells were washed and resuspended in cell lysis buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 %

Triton X-100, 10 % glycerol, 0.1 % SDS, 0.5 % sodium deoxycholate and protease inhibitors). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Total protein (10 µg) was resolved by SDS-PAGE in 4–12 % Bis-Tris Mini Gels and transferred to nitrocellulose membranes (NuPAGE, Life Technologies). Membranes were blocked in 3 % non-fat dry milk and incubated overnight at 4 °C with primary antibodies (Sigma-Aldrich): mouse monoclonal anti- α SMA (1:1,000, clone 1A4), mouse monoclonal anti-SM-MHC (1:500, clone hSM-V) or rabbit polyclonal anti-GAPDH (1:5,000). Membranes were then incubated for 1 h with goat anti-mouse and anti-rabbit HRP-conjugated secondary antibodies (Bio-Rad, Hercules, CA, USA). Detection was done with the ImmunStarTM WesternCTM Chemiluminescent Kit, and images were taken with a digital imaging system (ChemiDoc, Bio-Rad).

Statistics

Samples were compared using the Mann–Whitney-U-Test with the statistics software SPSS (SPSS Inc., Chicago, IL). The null hypothesis was rejected at p values of ≤ 0.05 .

Results

Immunohistochemistry of umbilical cord tissue

Endothelial cells from the umbilical vessels were stained by CD34 antibody. CD10 was exclusively expressed by stromal cells of Wharton's jelly, whereas the staining of anti-CD90, anti-vimentin, and anti- α SMA was additionally localized in umbilical blood vessels. The distribution of stromal cells with features of contractile cells was more restricted: desmin was preferentially expressed in the muscular and external layer of the umbilical cord vessels, as well as in stromal cells of the perivascular Wharton's jelly and the sub amniotic region (not shown). Expression of SM-MHC was localized in the tunica media of the umbilical vessels and their immediate environment (Fig. 1).

Characterization of HUASMC obtained by enzymatic digestion

Characterization by flow cytometry of cells freshly obtained by enzymatic digestion showed that, on average, 80 % of cells were CD31 and CD10 negative (data not shown). Dispase digestion after removal of the Wharton's jelly could further increase the purity of the population by 15 %. Flow cytometry of cell isolations derived from 11 umbilical cords indicated that 95 % of cells stained negative for CD31 and CD10 ($n = 11$, Fig. 2a, b). Western blot analysis confirmed the smooth muscle origin of the cells by positive reactions with anti- α SMA and anti-SM-MHC antibodies (Fig. 2c). Although isolated cells showed a high viability of 95 % microscopic observation of the cell populations after 24, 48 and 72 h indicated that 80–90 % of the cells failed to attach successfully (data not shown). Flow cytometry of the confluent cell population 3 weeks post isolation (P0) indicated an average of 85 % CD10+ cells, 5 % CD31+ cells and 5–10 % CD10–/CD31– cells. Similar proportions were maintained after one passage (P1). As shown in Table 1, these results could not be improved by using different protein coatings. The percentages of the CD10+ , CD31+ and CD10–/CD31– subpopulations relative to fibronectin, collagen I and gelatin coatings did not differ from the uncoated ones ($p > 0.05$).

CD29 was strongly expressed by HUASMC; it permitted gating blood cells out of the flow cytometry analysis and confirmed that no impairment of integrins occurred upon enzymatic treatment.

3D culture in MatrigelTM

In contrast to 2D culture, HUASMC could be cultured in MatrigelTM for several weeks. One week after embedding, cell clusters could be observed by live staining with calcein AM ($n = 3$, Fig. 3a). They kept mostly a round shape with minor lamellipodia formation (average spindled index of 1.25, following the method of Dikovsky et al. 2008). Then, slowly, clusters grew larger by sprouting to seek interconnections (average spindled index of 2.25, Fig. 3b). Cluster formation and rearrangement were probably due mainly to cell migration rather than cell duplication, since no evident increase in cell number was observed.

Fig. 1 Immunohistochemical staining of umbilical cord tissue. IgG control staining was documented as overview (a) showing an umbilical artery embedded in Wharton's jelly and (b) in detail at larger magnification. The dotted line marks the border between the Wharton's jelly and the vascular region. The endothelial marker CD34 is exclusively expressed by endothelial cells (c) CD10 antibody stains stromal cells outside the umbilical blood vessel (d) CD90 (e) vimentin (f) and α -SMA (g) are additionally localized in vascular regions, but only vimentin is expressed in endothelial cells. Desmin is preferentially expressed in the muscular and external layer of the umbilical cord vessels, as well as in stromal cells of the perivascular Wharton's jelly (asterisks, h), and the sub-amniotic region. SM-MHC is highly restricted to umbilical vessels. Except in endothelial cells, it is found in all layers of the umbilical vessels and in the cells of their immediate surrounding (asterisks, i). Scale bar 200 μ m; UCV, umbilical cord vessel; WJ, Wharton's jelly

reached after 3 weeks. Flow cytometry characterization of the cultures showed that in culture (P0) the percentage of CD10⁺/CD31⁺ cells was around 25 % and decreased to 10 % after one passage (P1). In P0 cultures, 70 % of the total population was represented by CD10⁺ cells. This value increased to 90 % in P1 (Table 1).

After two passages (P2), an average of 95 % of the cells was positive for CD10⁺ (Table 1). No expression of α -SMA and SM-MHC was observed in the cells cultured in SMC growth medium (Fig. 4a, b). Nevertheless, contractile protein expression was obtained after 6 days in Medium 231 supplemented with SMDS. Figure 4c, d show immunofluorescence staining of α -SMA and SM-MHC after incubation in differentiation medium.

During culture, cells did not cause any compaction of the gel, therefore the construct kept its dimensions.

Characterization of the cells obtained by the explant method

Cells started outgrowing the explants and colonizing the well bottom 1 week after plating. Confluence was

Discussion

Key vascular inflammatory processes related to the shift of VSMC phenotype are fervently under investigation because of their role in pathologies such as atherosclerosis. In vitro experiments are an essential step in vascular biology research and there is a

Fig. 2 Flow cytometry characterization of the cells obtained by enzymatic digestion derived from 11 umbilical cords shows that on average 95 % cells are CD10⁺/CD31⁺, 4 % are CD10⁺, 1 % are CD31⁺ (a, n = 11). In detail, analysis of two

representative cell batches with flow cytometry (b) and western blotting (c) showing that CD10⁺/CD31⁺ cells are SM-MHC and α -SMA positive

Table 1 Distribution of the subpopulation percentages obtained by the enzymatic and the explant method

P0		CD10+	CD10–/CD31–	CD31+	No. of batches
Enzymatic	Uncoated	85.9 ± 4.4	10.8 ± 2.0	4.0 ± 4.0	3
	Fibronectin	88.7 ± 9.7	4.6 ± 3.3	6.7 ± 7.0	3
	Collagen I	86.1 ± 18.9	12.9 ± 17.8	1.1 ± 1.1	2
	Gelatin	85.7 ± 6.0	9.1 ± 3.4	3.9 ± 3.7	3
Explant		72.2 ± 12.7	26.8 ± 13.4	0.1 ± 0.1	4
P1					
Enzymatic	Uncoated	88.1 ± 10.2	11.9 ± 10.2	0.0	2
	Fibronectin	84.2 ± 22.3	14.6 ± 20.5	1.3 ± 1.8	2
	Collagen I	90.0 ± 13.4	9.9 ± 13.3	0.2 ± 0.1	2
	Gelatin	99.8	0.2	0.0	1
Explant		87.5 ± 6.5	12.4 ± 6.5	0.1 ± 0.1	4
P2					
Explant		98.0 ± 2.2	2.0 ± 2.0	0.0 ± 0.0	2

Fig. 3 Calcein AM staining of HUASMC embedded in Matrigel. In the first 2 weeks (a) cells remained mostly rounded and form minor aggregates. After 1 month of culture (b) cell clusters were larger and sprouted to connect with other clusters or other cells. Scale bar 150 µm

requirement for well-characterized cell cultures. Because of the particular origin of HUASMC with respect to other VSMC from adult tissue, we asked ourselves if the current characterization marker panel is suitable for their isolation and culture.

Immunohistochemical staining of umbilical artery sections showed that HUASMC and pv-WJSC share the expression of markers commonly used for VSMC characterization: α -SMA, SM-MHC, desmin and vimentin. In accordance with previous literature (La Rocca et al. 2009; Farias et al. 2011), our results confirmed that α -SMA, vimentin and desmin are expressed by both cell types and, therefore, are unsuitable for specific characterization. Moreover, SM-MHC, often regarded as the most specific marker of VSMC, is also expressed by some cells of the perivascular region of Wharton's jelly, which is consistent with the results reported by Nanaev et al.

(1997). To identify HUASMC by negative staining, we investigated the expression of CD90 and CD10, both being mesenchymal markers. In particular, CD90 is also commonly used in VSMC characterization to identify fibroblast contamination (Leik et al. 2004), although its expression on VSMC subtypes has been reported (Kisselbach et al. 2009). According to our analysis, CD90 is expressed by stromal cells of the medial layer of the umbilical arteries, but the staining of VSMC subtypes cannot be excluded. In conclusion, among the investigated markers, only CD10 could segregate pv-WJSC from HUASMC. Therefore, we decided in favor of a method based on the negative staining for pv-WJSC and endothelial cell-specific markers CD10 and CD31, respectively. The reliability of considering double negative cells (CD10–/CD31–) as HUASMC was confirmed by analysis of α -SMA and SM-MHC expression by western blotting.

Fig. 4 Staining for α -SMA (a, c) and SM-MHC (b, d) was negative in cells in P2 from the explant method (95 % CD10+) cultured in SMC growth medium (a, b) and positive after 6 days in Medium 231 supplemented with SMDS (c, d). Scale bar 50 μ m

According to our characterization criteria (α -SMA+ , SM-MHC+ , CD10–/CD31–, CD34–), we could prove the enzymatic harvesting of HUASMC as a preferential isolation method (95 % pure HU-ASMC population). The results obtained with the explant method (25 % purity) indicated that pv-WJSC had presumably a higher migration capability than HUASMC. Consequently, a pure population may only be obtained by adding an additional step to sort CD10 positive cells out.

If we compare P0 or P1 of both methods there is no significant difference in the percentage of CD10 positive cells. The high population purity obtained after isolation by enzymatic digestion could not be maintained in culture due to poor attachment and slow proliferation of HUASMC, which led to an increase of contamination by CD10 positive cells. Attachment impairments in freshly isolated HU-ASMC and myoblasts are well reported in literature (Rensen et al. 2007; Pavlath et al. 2005). But, in contrast to Rensen et al. (2007), we could not see any improvement by the use of collagen coating. We could not find any evidence of dedifferentiation of HUASMC to a mesenchymal phenotype or differentiation of HUASMC from a contractile to a proliferative phenotype, which is described in

literature as well (Rensen et al. 2007). We could confirm that purity loss was due to the low attachment of the cells, investigated by microscopic analysis of the population. This low adhesion was not caused by integrin damage, as integrin β 1 (CD29), predominant in vascular smooth muscle (Moiseeva 2001), was expressed on the cell surface (Fig. 4). Since pv-WJSC did not show such an adhesion deficiency, it is unlikely that collagenase treatment compromised integrin function. Impairment of adhesion and spreading was probably due to the lack of a suitable matrix environment, since cells could adhere and remain viable for several weeks when embedded into 3D structures. This was observed in both 3D matrices, MatrigelTM and collagen type I gels. HUASMC embedded in MatrigelTM retained their round shape, probably due to the elastic modulus of the gel, which is approximately 450 Pa at 37 °C (Soofi et al. 2009). In fact, similar to other cell types, VSMC have been reported to sense the stiffness of the matrix in which they are embedded. Cell spreading has been observed when the elastic modulus ranges between 10 and 100 Pa; however, for moduli above 500 Pa cells tend to keep a round shape (Dikovskiy et al. 2008). Similar results were obtained in studies using

bladder SMC (Adelow et al. 2008), where cells showed considerably less spreading in gels stiffer than 192 Pa.

Unlike HUASMC, pv-WJSC could be cultured in uncoated 2D plates and showed a long spindle morphology like VSMC. Under standard culture conditions, pv-WJSC did not express α -SMA and SM-MHC. After treatment with differentiation medium, cells increased their expression of both contractile proteins and simultaneously acquired a wide, flat morphology; very similar to the one described by Karahuseyinoglu et al. (2007). It is well known that in vitro culture of VSMC promotes a switch from a contractile (quiescent) phenotype to a more secretive (proliferating) one. Cells in the latter phenotype are characterized by a higher proliferation ratio, motility, extracellular matrix production and down-regulation of contractile protein expression. Reversion of VSMC towards a contractile phenotype in vitro is obtained by serum starvation or treatment with heparin-supplemented medium (Rensen et al. 2007; Stegemann et al. 2005). Similarly to VSMC, we have shown that pv-WJSC (CD10+) increased expression of α -SMA and SM-MHC by the use of the same stimulants, and therefore, characterization of HUASMC in culture cannot be limited to the analysis of contractile protein expression.

In this work we have shown that isolation of HUASMC populations with high purity is possible and 3D culturing improves attachment of isolated cells. HUASMC in 3D matrices were proven to be viable and functional. Nevertheless, SMC growth medium could not induce visible proliferation and the effect of further growth factors (e.g. Transforming Growth Factor (TGF)- β ; Shi et al. 2014) should be investigated. Perivascular-WJSC and VSMC show many common phenotypic features with respect to morphology and protein expression, which pose a challenge to the detection of pv-WJSC contamination. Therefore, we highly suggest the analysis of endothelial markers and the mesenchymal marker CD10 for the accurate characterization of HUASMC.

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