Resident phenotypically modulated vascular smooth muscle cells in healthy human arteries

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Abstract

Vascular interstitial cells (VICs) are non-contractile cells with filopodia previously described in healthy blood vessels of rodents and their function remains unknown. The objective of this study was to identify VICs in human arteries and to ascertain their role. VICs were identified in the wall of human gastro-omental arteries using transmission electron microscopy. Isolated VICs showed ability to form new and elongate existing filopodia and actively change body shape. Most importantly sprouting VICs were also observed in cell dispersal. RT-PCR performed on separately collected contractile vascular smooth muscle cells (VSMCs) and VICs showed that both cell types expressed the gene for smooth muscle myosin heavy chain (SM-MHC). Immunofluorescent labelling showed that both VSMCs and VICs had similar fluorescence for SM-MHC and αSM-actin, VICs, however, had significantly lower fluorescence for smoothelin, myosin light chain kinase, h-calponin and SM22α. It was also found that VICs do not have cytoskeleton as rigid as in contractile VSMCs. VICs express number of VSMC-specific proteins and display features of phenotypically modulated VSMCs with increased migratory abilities. VICs, therefore represent resident phenotypically modulated VSMCs that are present in human arteries under normal physiological conditions.

Keywords: vascular interstitial cell ● vascular smooth muscle cell ● human ● phenotypically modulated vascular smooth muscle cells ● gastro-omental arteries ● filopodia ● budding

Introduction

The primary function of vascular smooth muscle cell (VSMC) in adult animal is contraction which is necessary for maintaining vascular tone and control of the blood flow. In contrast with skeletal and striated muscle cells, VSMCs are not terminally differentiated and can change their phenotype in response to environmental cues. The phenotypic modulation or "switch" in VSMCs is accompanied by accelerated migration, proliferation and production of extracellular matrix components [1]. In such state, VSMCs are referred to as "synthetic" or phenotypically modulated VSMCs (PMVSMCs). It is established that PMVSMCs play a key role during vascular injury and other pathological conditions of blood vessels. The latter includes a number of proliferative cardiovascular diseases such as atherosclerosis, hypertension, diabetic vascular complications and restenosis after angioplasty or bypass [2]. It is believed that contractile VSMCs either proliferate at very low rate or do not proliferate at all as the vast majority of VSMCs observed in mitosis were at least partially modulated towards the "synthetic" state containing few myofilament bundles and a large number of organelles such as free ribosomes and rough endoplasmic reticulum [3]. These observations suggest that phenotypic modulation from the contractile towards the synthetic state is a necessary condition for a proliferation of VSMCs in blood

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vessel. The change in the state of VSMCs from contractile to synthetic phenotype is accompanied with a decrease in expression of VSMC-specific proteins, such as αSM-actin, smooth muscle myosin heavy chain (SM-MHC), SM22α, h1-calponin, smoothelin, caldesmon and telokin [1]. Most importantly, all these proteins, except SM-MHC, can be expressed, at least transiently, by other types of cells of the blood vessel wall [2]. This makes unambiguous identification of PMVSMCs very difficult. Also, due to substantial heterogeneity within the population of VSMCs in disease and the absence of efficient specific markers for PMVSMCs or VSMCs at various stages of phenotypic modulation, it is very difficult to obtain "pure" samples of PMVSMCs. This impedes the employment of efficient molecular biology techniques that could lead to either establishing specific transcription mechanisms governing phenotypic modulation *in vivo*, or identification of highly specific markers for PMVSMCs in disease. Because of above reasons, the majority of the studies of PMVSMC in pathology relies on immunofluorescent labelling using VSMC-specific markers in multicellular preparations or using of the culture of VSMCs as a closest surrogate for *in vivo* phenotypic modulation of VSMCs.

Recently, the presence of a new type of cell, called vascular interstitial cells (VICs), was reported in various blood vessels of rodents, including veins and arteries [4–7]. These non-contractile cells often had an irregularly shaped body and displayed the presence of the multiple filopodia. Vascular interstitial cells were observed under normal physiological conditions either after dispersal of the blood vessels by proteolytic enzyme treatment or in the wall of blood vessels using transmission electron microscopy (TEM) suggesting that their appearance is not an artefact of cell isolation [4, 6, 7]. The population of these cells in blood vessels from animals could be quite substantial; their number can reach up to 5% of total number of contractile VSMCs in the dispersal of various blood vessels [6]. Freshly dispersed VICs from animal blood vessels expressed smooth muscle myosin heavy chain (SM-MHC), which is a specific marker for the VSMC phenotype [7, 8]. This suggests that VICs and VSMCs belong to the same type of cells. Vascular interstitial cells were identified in number of blood vessels from rodents and it was proposed that VICs can be present in all blood vessels [6]. In this study, we established that VICs are also present in human blood vessels. We also addressed the important questions whether human VICs share the properties of VICs previously described in animal vasculature and whether these cells demonstrate the features of the putative phenotypically modulated VSMCs. The preliminary account of this study was presented in an abstract form at Atherosclerosis, Thrombosis and Vascular Biology 2011 Scientific Sessions.

Materials and methods

Vascular tissue retrieval

This investigation conforms to the principles outlined in the Declaration of Helsinki and was approved by the local Research Ethics Committee (09/H0803/103) for retrieval of human blood vessels. In this study, we used samples obtained from 12 non-diabetic and non-hypertensive patients (3

males and 9 females, average age 56 ± 4 years) undergoing abdominal surgery. Prior to surgery, the consents were obtained from patients for using of small fragment of healthy visceral adipose tissue. The adipose tissue containing branches of human gastro-omental arteries was dissected during surgery, and immediately placed into ice-cold (4°C) physiological saline solution (PSS) of following composition (mmol/l): KCl 6, NaCl 120, MgCl$_2$ 1.2, CaCl$_2$ 2.0, D-glucose 10 and HEPES 10; pH was adjusted to 7.4 with NaOH. The samples were kept in ice-cold PSS and dissected in the laboratory within 1 hr after dissection. Arteries were identified as the muscular vessel running as a pair with thin walled veins. The small (normally smaller than 700 μm in diameter) branches of gastro-omental arteries were thoroughly cleaned of fat and connective tissue and used in experiments. Some fragments of blood vessels from every patient were fixed and their cross-sections were later examined under light microscope. All samples from patients displaying any visible alterations of the vessel wall such as presence of atherosclerotic lesions were not included in the study. Samples of cross-sections of omental tissue stained with haematoxylin and eosin and displaying general architecture of arteries are shown in Figure S1 in Supporting Information.

Single cell isolation

Single cells were obtained by enzymatic dispersion of fragments of arteries as we described previously [9]. Briefly, fragments of blood vessels were cleaned of fat and connective tissue were incubated in Ca^{2+}-free PSS containing 1 mg/ml collagenase type IA, and 0.5 mg/ml thermolysin type X (both Sigma-Aldrich, Poole, UK) for 30 min. at 37°C following 5 min. wash in enzyme-free solution. The single cells were obtained by gentle agitation with Pasteur pipette and then allowed 15 min. to attach to glass coverslip forming the bottom of experimental chamber. The Ca^{2+} concentration in chambers was gradually increased to 2 mmol/l which prevented the myocytes from 'calcium shock' and hypercontraction [10]. After isolation, cells were either collected within 3 hrs for molecular biology experiments, or fixed within 1 hr for immunocytochemical labelling.

Scanning and transmission electron microscopy

Scanning and transmission electron microscopy was preformed as described in our previous studies [7] and described in detail in supplementary methods.

Reverse trancription polymerase chain reaction

For RT-PCR, isolated VSMCs and VICs (~150 cells each) were collected separately using a glass pipette attached to micromanipulator (Supporting Information Video S1) and frozen on dry ice immediately. Total RNA was extracted using RNeasy extraction kit (Qiagen, Crawley, UK) and cDNA was obtained using Superscript II Reverse Transcriptase (Invitrogen, Paisley, UK) and used in PCR. cDNA was used as a template for PCR reaction using platinum *Taq* DNA polymerase (Invitrogen). Amplification was performed according to the following schedule using a T3 Thermocycler (Biometra, Germany): 94°C for 2 min.; 40 cycles of 94°C for 30 sec.; 57°C for 60 sec.; and 72°C for 3 min., followed by a final elongation period of 10 min. at 72°C. No-template control PCR was also

performed simultaneously with every reaction. Primers were designed so that they spanned at least one intron of the genomic sequence to avoid detecting genomic DNA contamination. The experiments were repeated with three preparations from different patients of independently collected VICs and VSMCs. The primers were designed to amplify the genes encoding proteins of interest. The PCR products were separated and visualized in ethidium bromide-stained 2% agarose gel by electrophoresis.

Immunofluorescent analysis

Immunofluorescent analysis was performed as described previously [7, 8]. Single cells were fixed with 4% formaldehyde solution at 4°C for 4 min., washed with PSS and incubated with PSS containing 2% bovine serum albumin (BSA) and 0.3% Triton X-100. Then, samples were incubated with primary antibodies in PSS containing 2% BSA overnight at 4°C, washed with PSS and incubated for 2 hrs with secondary antibodies conjugated with fluorescent probes. Samples were washed with PSS, and viewed using the laser scanning confocal microscope Zeiss LSM 510 (Zeiss, Oberkochen, Germany). For the list and dilutions of the antibodies used, see online data supplement.

Confocal imaging data were processed and analysed using Zeiss LSM software. An image taken approximately in the middle of the cell was selected out of the z-stack of horizontally taken images [8]. Using such an image the average pixel fluorescence (APF) was calculated according to equation:

$$AFP = \frac{\sum i(p)}{n(p)} \text{ (intensity units/pixel)}$$

where $i(p)$ is the intensity of a pixel within the confocal plane of the cell, and $n(p)$ is the total number of pixels of the plane. Average pixel fluorescence values obtained from each VIC and each VSMC were normalized to the mean APF value derived from all VSMCs in each experiment (sample). The collective means for VICs and for VSMCs for each protein from different samples obtained from different patients were then calculated and the differences statistically compared. Such approach allowed to visually demonstrate the difference in the expression of markers between two cell types in data summarized from more than one patient and to diminish any possible variation in conditions of experiments. Statistical evaluation and graphs were created using Origin (OriginLab, Northampton, MA, USA) and final images were produced using Corel Draw 7.

Statistical analysis

All data is shown as a mean ± SEM for the number of cells (n) analyzed. Statistical significance was calculated using Student's t-test for unpaired observations and the differences where $P < 0.05$ were considered significant.

Materials

All general chemicals including proteolytic enzymes were purchased from Sigma-Aldrich. Molecular biology reagents and primers were purchased from Invitrogen, apart from RNeasy extraction kit which was purchased from Qiagen. All primary antibodies were purchased from Abcam (Cambridge, UK) and secondary antibodies were purchased from Mobitec (Göttingen, Germany). BODIPY 558/568 phalloidin was purchased from Invitrogen.

Results

Morphofunctional characteristics of VICs

The enzymatic dispersion of small human gastro-omental arteries resulted in cell suspension containing contractile VSMCs and cells with morphological characteristics similar to VICs previously described in animal blood vessels (Fig. 1A and B). Vascular interstitial cells were observed in every dispersal of human arteries as cells with filopodia that often had a multi-polar body (Fig. 1B). Freshly dispersed VICs from human arteries were also non-contractile: in contrast with VSMCs they did not contract in response to application of 10 mmol/l caffeine which at this concentration acts as a very potent spasmogen. In contrast, contractile VSMCs always contracted in response to application of the drug (Supporting Information Figure S1). To confirm the presence of VICs in the wall of human arteries we employed TEM. These experiments revealed that cells with similar morphological characteristics are present within the wall of blood vessels thus, confirming that
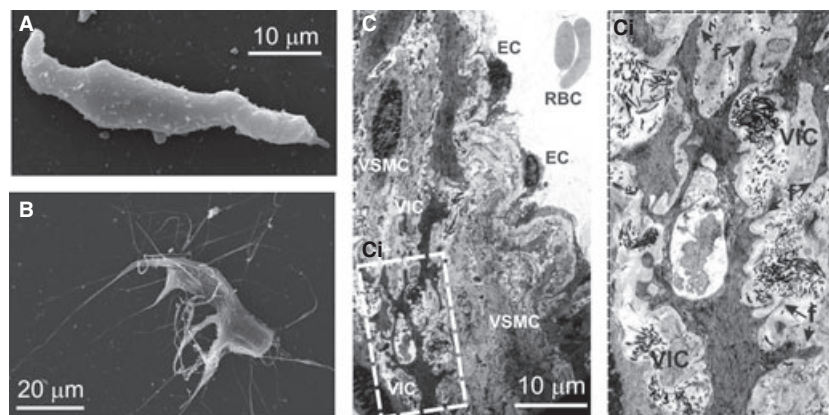

Fig. 1 Identification of VICs in small branches of human gastro-omental artery. Scanning electron micrographs of single VSMC (**A**) and VIC (**B**) obtained by enzymatic dispersion. Note the absence of filopodia in VSMC. (**C**) Transmitted electron micrographs reveal VICs as darker irregularly shaped cells with numerous filopodia in the wall of the vessel. (**Ci**) shows enlarged image of boxed region in (C) at higher magnification. RBC: red blood cell; EC: endothelial cell.

appearance of freshly isolated human VICs is not an artefact of enzymatic dispersion procedure (Fig. 1C). Further TEM study of VICs and VSMCs revealed that VICs in human gastro-omental arteries form numerous cell-to-cell contacts with adjacent VSMCs and have significantly larger number of calvolae in comparison with VSMCs, particularly where sides of VIC were facing sides of VSMCs (Fig 2Ii–1iii). In addition, the size of the dense bodies in VICs normally did not exceed 1 μm in its highest x–y dimension, whereas in VSMCs the dense bodies looked more contrast and some of these could exceed more than 3 μm in its highest x–y dimension.

Observation under bright field microscope showed that VICs from human arteries actively changed their shape within the first hour after cell isolation. These changes included the extension of existing filopodia and the formation of new filopodia, as well as expanding and retracting of the cell body (Fig. 3, Supporting Information Video S2). It is noteworthy, that the speed and occurrence of such changes in VICs decreased with time, and usually little changes were observed after 2 hrs after isolation. Most importantly, VICs which have formed bud-like structures from their filopodia were occasionally observed in cell dispersal within the same time interval (Fig. 4). Similar structures are observed *in vitro* and *in vivo* in sprouting endothelial cells during angiogenesis and their appearance indicates the cellular migration leading to formation of new endothelial cells [11, 12]. In addition, newly formed filopodia were observed in bud-like structures (Fig. 4iii) further suggesting that VICs have higher migratory abilities compared with those of VSMCs as an abundance of the filopodia on sprouting endothelial cells or on axonal growth cone is an indicative of the active migratory phenotype [13–15]. In contrast, contractile VSMCs during the same period of observation did not demonstrate significant changes of their body (Supporting Information Figure S3), although some contraction of VSMC towards the central part of the cell was occasionally observed. This was likely caused by an increase in extracellular calcium concentration after isolation (see Methods).

Expression of lineage markers in VICs from human vessels

To confirm the type of cell to which VICs from human arteries belong to, we employed RT-PCR analysis using cDNA obtained from separately collected VSMCs and VICs (~150 cell each type) (Fig. 5). Similar to VSMCs, VICs showed the expression of SM-MHC, which is a highly specific marker for smooth muscle type of cell [1], suggesting that VICs represent phenotypically modulated VSMCs. VICs did not express genes for other types of cell that can be found in the vascular wall such as neurons, fibroblasts, pericytes, endothelial cells or macrophages. VICs did not express c-kit gene encoding stem cells factor protein, previously reported in wall of various, including human, blood vessels [16–20]. VICs also did not express the gene for another stem cell marker, CD133, which is expressed in endothelial progenitor cells [21] and detected in human progenitor cells of atherosclerotic lesions [22].
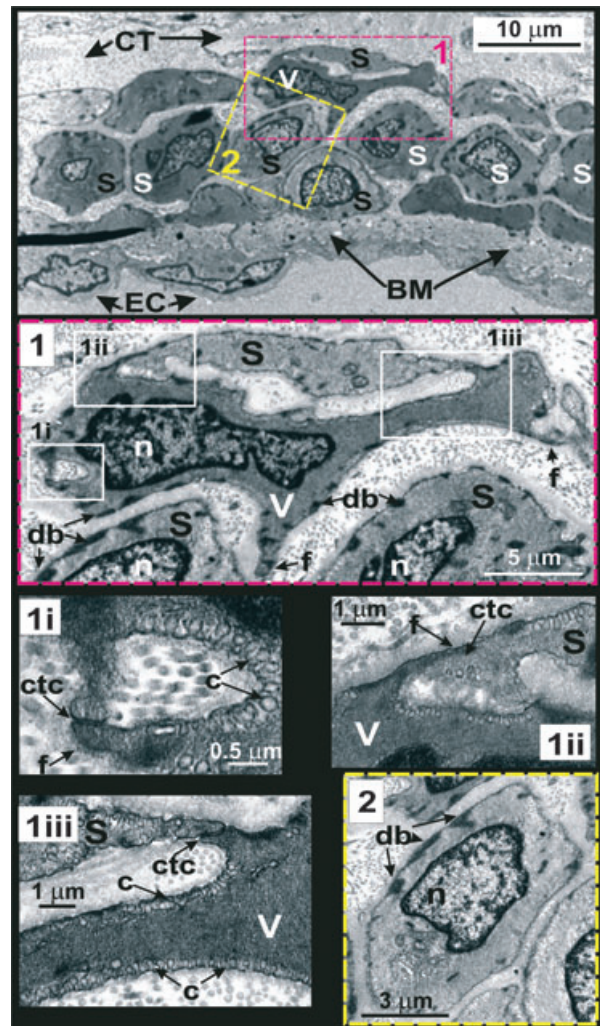

Fig. 2 Transmission electron microscopy (TEM) characterizations of VICs and VSMCs in the wall of human gastro-omental arteries. Approximate cross-sections of blood vessels. Top panel shows the architecture of the blood vessel; (s) depicts VSMC, (V) – VICs, (EC) – endothelial cells, (BM) -basal elastic membrane separating endothelium and smooth muscle layers, (CT) – connective tissue in adventitial layer of the blood vessel. VIC depicted by dashed pink rectangle is shown enlarged in (**1**). (n) indicates nucleus, (db) – dense bodies, (f) – filopodia. VIC forms tight contacts (ctc) with VSMCs (enlarged in **1i-1iii**) and significantly larger number of caveolae (c) compared to VSMCs which is shown enlarged in (**2**).

Expression VSMC-specific proteins in VICs

It is considered that traditional PMVSMC are characterized by decreased expression in a number of VSMC-specific proteins. As it was impossible to obtain sufficient amount of material from manually collected freshly isolated cells to analyse the expression of proteins using the western blot technique, we analyzed the expression of
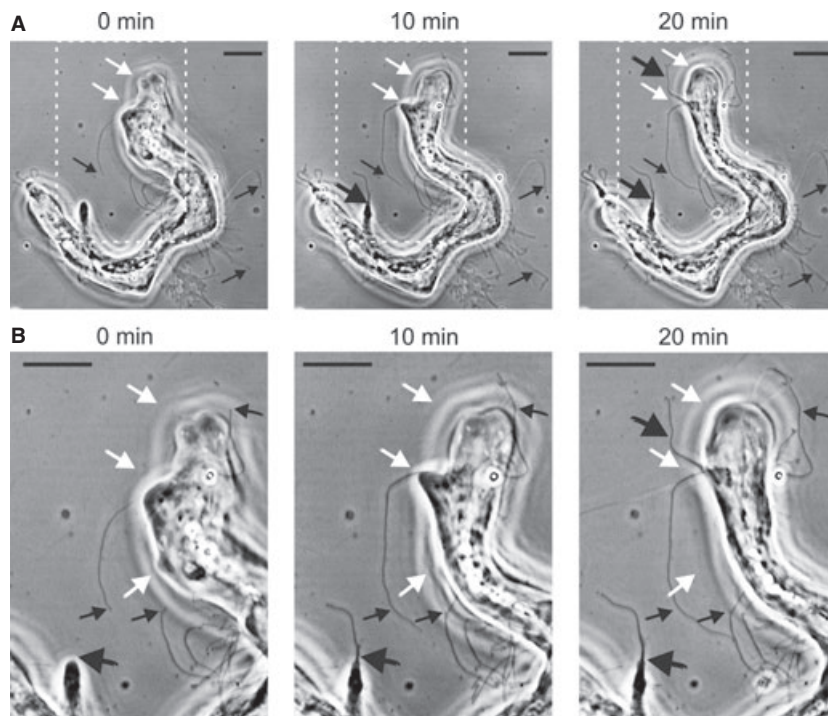
Fig. 3 VICs actively change their shape, form new and extend existing filopodia after isolation. (**A**) shows VIC during 20 min. of live imaging at room temperature. Small black arrows indicate the position of the tips of filopodia at the start of the recording, white arrows point to the parts of the cell body, which display change in shape. Large black arrows indicate formation of new filopodia. (**B**) shows enlarged region of VIC depicted by white dashed rectangle in (A) shown at higher magnification. Horizontal bar corresponds 10 μm.

VSMC-specific proteins in both freshly isolated VICs and VSMCs using fluorescent immunocytochemistry approach. The analysis of fluorescence in both types of cells showed that VICs and VSMCs displayed similar mean pixel fluorescence for SM-MHC ($90 \pm 7\%$ in VICs to that of VSMCs, $P > 0.05$; 31 VSMCs, 28 VICs; from 3 patients), αSM-actin ($93 \pm 7\%$ in VICs, $P > 0.05$; 31,29;3), myosin light chain (MLC) ($99 \pm 10\%$ in VICs, $P > 0.05$; 32,32;3) and h-caldesmon ($94 \pm 8\%$ in VICs, $P > 0.05$; 27,22;2), (Fig. 6A). VICs, however, had significantly lower fluorescence for smoothelin ($33 \pm 3\%$ in VICs, $P < 0.001$; 33,31;3), myosin light chain kinase (MLCK) ($27 \pm 3\%$ in VICs, $P < 0.001$; 28,22;2) and calponin (0.13 ± 0.03 in VICs, $P < 0.001$; 33,31;3). Very little of fluorescence was observed for SM22α (0.04 ± 0.01 in VICs, $P < 0.001$; 34,34;3). The latter four proteins are considered as markers of mature VSMC [23–27], suggesting that VICs may represent de-differentiated or immature VSMCs.

The presence of stress fibres in VICs from human arteries

During the manual collection of isolated cells with a glass micropipette for molecular biology experiments (see Methods), it was observed that VICs do not have cytoskeleton as rigid as in VSMCs. Thus, VICs easily passed into the pipette with the diameter of the tip less than half of the diameter of these cells (Supporting Information Figure S4. A1–3, Video S3). In contrast, VSMCs similar in size to VICs occluded pipette with the same size orifice (Supporting Information Figure S4. B1–3,

Video S3). These observations suggest that the organisation of cytoskeletal proteins in VICs is different from that in VSMCs.

It is considered that αSM-actin is the most abundant contributor to the cytoskeleton of VSMC [1]. The changes in assembly and disassembly of another cytoskeletal protein, f-(fibrillar)-actin, are considered as the driving process behind phenotypic changes of VSMC [28]. Therefore, a spatial distribution of these two cytoskeletal proteins was assessed in VICs and VSMCs using high-resolution laser scanning confocal microscopy. For this purpose fixed preparation of freshly isolated cells were labelled with the fluorescent probe BODIPY 558/568 phalloidin to f-actin and with a specific antibody to detect αSM-actin. These experiments showed that in human arterial VICs, αSM- and f- actins are organized into strands running along the cell's axis (Fig. 7B and D). In contrast, in contractile human VSMCs, F- and αSM- actins were more densely packed and it was impossible to identify individual strands at the same or higher resolution of the confocal microscope (Fig. 7A and C).

Discussion

During the last several years we have observed that VICs are present under normal physiological conditions in all blood vessels [6]. These included small arteries such as resistance mesenteric, kidney or cerebral arteries from mouse, rat, guinea pig and rabbit, as well as large vessels such as rat or mouse mesenteric artery and aorta or rabbit portal vein. In this study, we confirmed the presence of such cells under normal conditions in small branches of human gastro-omental

Fig. 4 Sprouting VIC in cell dispersal from human arteries. The transmitted light image shows sprouting VIC in cell dispersal from human gastro-omental artery. Insets (**i**), (**ii**) and (**iii**) show enlarged regions of VIC depicted by dashed rectangles. White arrows indicate formations of buds from filopodia, black arrows indicate the presence of new filopodia in buds.
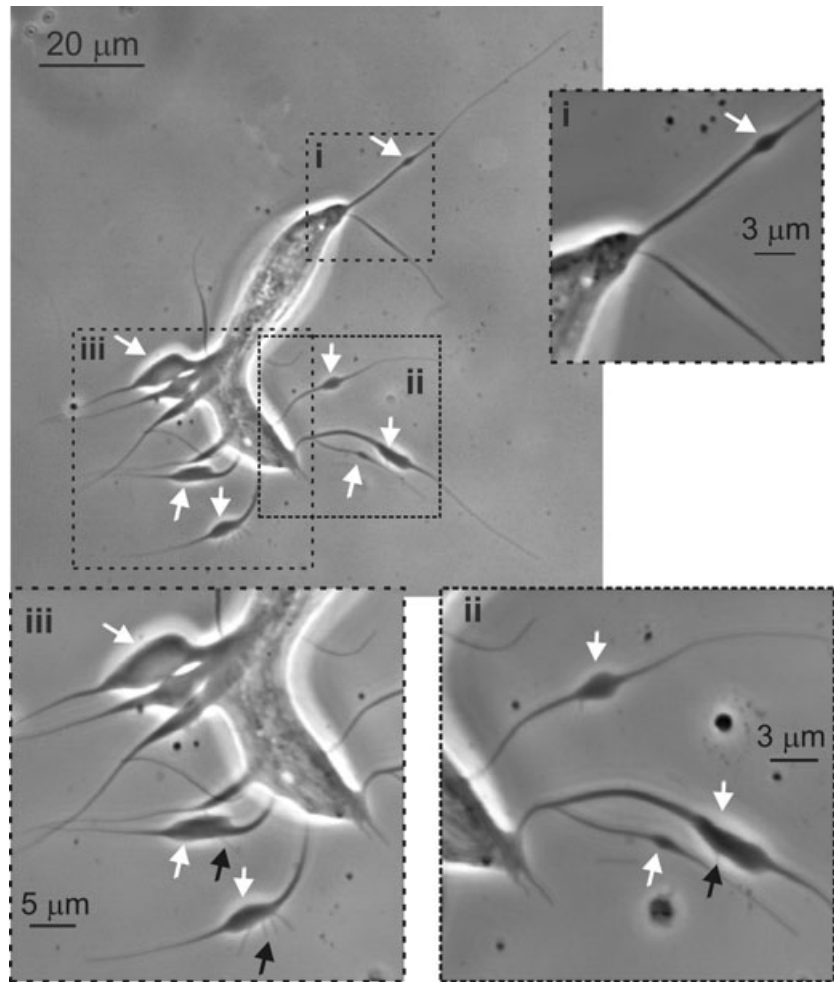
artery. These findings allow us to assume that such cells are present in all major mammalian blood vessels, although the presence of VICs is not confirmed in smaller blood vessels such as arterioles. VICs from various vascular beds express specific marker of contractile VSMCs, SM-MHC, and do not express markers of other cell types present in the wall of blood vessels [8], suggesting that VICs represent phenotypically modulated VSMCs [7]. VICs from all blood vessels from different species have at least four major distinct morpho-functional features which differ them from contractile VSMCs: (1) they are non-contractile; (2) they have multiple filopodia and often irregularly-shaped multipolar body; (3) VICs form new and extend existing filopodia, and actively changing the shape of their bodies after isolation, whereas a shape of the contractile VSMCs remains relatively static during same period of observation; (4) VICs do not have a cytoskeleton as rigid as in contractile VSMCs and their cytoskeleton has well-defined dense actin stress fibres. Such stress fibres normally observed in VSMCs only after several days in the culture [29]. VICs also showed decreased expression of number of VSMC-specific proteins including smoothelin and SM22α which were reported as indica-

tors of phenotypic modulation of mature/contractile VSMCs *in vitro* and disease [26, 30–33]. These observations allow us to suggest that VICs are a specialized group of phenotypically modulated or de-differentiated VSMCs that are constitutively present in all major blood vessels and share similar morphological and likely functional properties.

Our TEM experiments showed that VICs have significantly larger number of caveolae than VSMCs which is similar to our previous TEM observation of VICs and VSMCs from rat cerebral arteries [7]. Interestingly, we observed that in VICs from rat mesenteric arteries the gene expression for caveolin-1, which is normally localized to caveolae in VSMCs [34], was significantly (hundred times) higher than in contractile VSMCs (unpublished observation), suggesting that such difference in caveolae expression can possibly be attributed with VICs in all blood vessels. Notably, the opposite situation was reported in cultured VSMCs where the number of caveolae was found to be distinctly reduced when smooth muscle cells shift from a contractile to a synthetic phenotype [35]. In addition, we observed that size of dense bodies, which are normally associated with structural proteins such as actin and myosin, in VICs normally did not exceed 1 μm, whereas
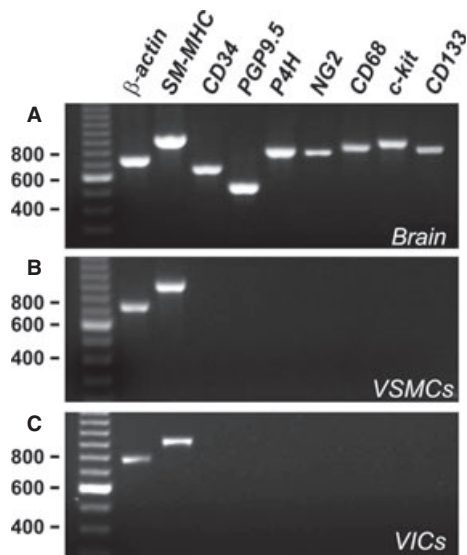
Fig. 5 RT-PCR from separately collected VICs and VSMCs. The following primers were designed to amplify genes associated with certain cell types: SM-MHC (SMCs), CD34 (fibroblasts and ECs), PGP9.5 (neurons), P4H (fibroblasts), NG2 (pericytes), CD68 (macrophages), c-kit (interstitial cells of Cajal), cd133 (stem cells). β-actin was used as a positive control. (**A**) primers specificity was tested using human brain cDNA. (**B**), (**C**), VICs and VSMCs showed the presence of SM-MHC suggesting that VICs belong to VSMC type.

in VSMCs dense bodies exceeding 3 μm were observed. This characteristic of VICs could be similar to that reported in synthetic myocytes where thick myofilaments observed in contractile VSMCs were no longer observed in myocytes after 7 days in the culture [36].

It is traditionally accepted that synthetic smooth muscle cells are characterized by downregulation in expression of smooth muscle specific proteins compared to contractile VSMCs [1, 37, 38]. It is noteworthy, that majority of these data were obtained using the culture of VSMCs. Our immunocytochemical experiments showed that although some proteins such as calponin, sm22α, MLCK and smoothelin were downregulated in VICs, the expression of two major VSMC-specific proteins, αSM-actin and SM-MHC, were not downregulated compared to contractile VSMCs. These observations may suggest that: (1) VICs cannot be detected in the blood vessel wall using most common markers such αSM-actin and SM-MHC (2) mechanisms governing phenotypic modulation of VICs (in vivo) are likely different from those for synthetic VSMCs in the culture (in vitro).

Polymerized actin in migrating cells is organized in three major subtypes: stress fibres, lamellipodia and filopodia. These contractile structures are thought to generate driving forces forwarding movement of the cell [39]. Freshly dispersed VICs from human arteries show the presence of actin stress fibres and filopodia, whereas contractile VSMCs do not have such features. Moreover, an abundance of the filopodia on sprouting of endothelial cells or on axonal growth cone is an indicative of the active migratory phenotype [14, 15]. In addition, migrating VSMCs during remodelling of blood ves-
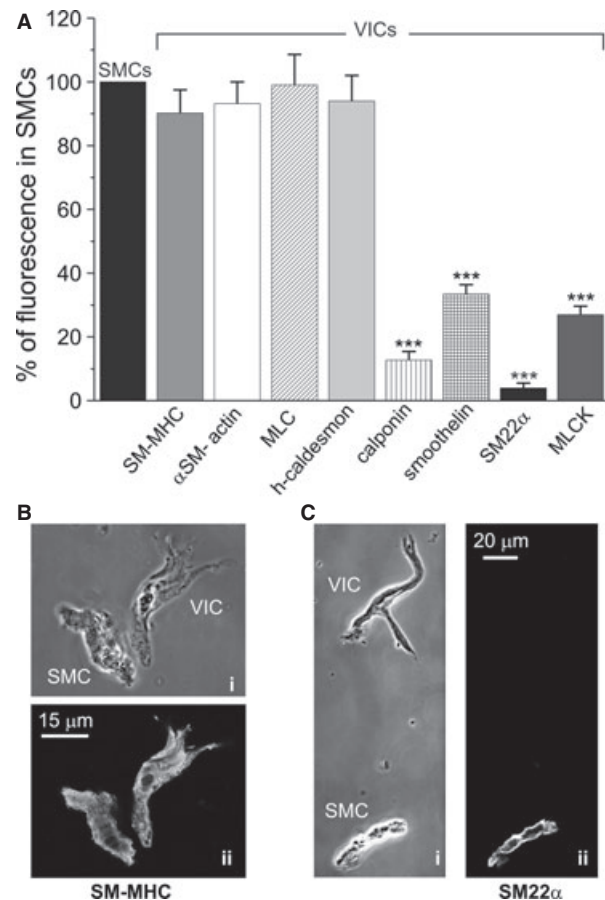
Fig. 6 Immunofluorescent analysis of VSMC-specific proteins expression in VSMCs and VICs. (**A**) graph of mean pixel fluorescence in VICs normalized to its value in VSMCs. The following proteins were detected: SM-MHC (31 VSMCs, 28 VICs; from 3 patients) αSM-actin (31,29;3), MLC (32,32;3), h-caldesmon (27,22;2), calponin (33,31;3), smoothelin (33,31;3), SM22α (34,34;3), MLCK (28,22;2). ***- indicates $P < 0.001$. (**B**) shows representative images of isolated cells labelled for SM-MHC (**B**) and SM22α (**C**). **i** – indicates transmitted light, **ii** – fluorescent images.

sels such as coronary artery VSMCs in restenosis, change their cell shape and form filopodia. [40]. One of the most important features observed in VICs from human blood vessels is an ability to change the shape of their body after enzymatic dispersion of blood vessels wall. The growth of new and the extension of existing filopodia as well as expanding and retracting of the body of the freshly isolated cell (Supporting Information Video S1) are the processes that undoubtedly characterises VICs as PMVSMCs with increased migratory ability compared to that of VSMCs. We also observed newly formed filopodia in sprouting of VICs (Fig. 4iii) that further confirms that VICs have higher migratory abilities compared to those of VSMCs as an abundance of the filopodia on sprouting endothelial cells or on axonal growth cone is an indicative of the active
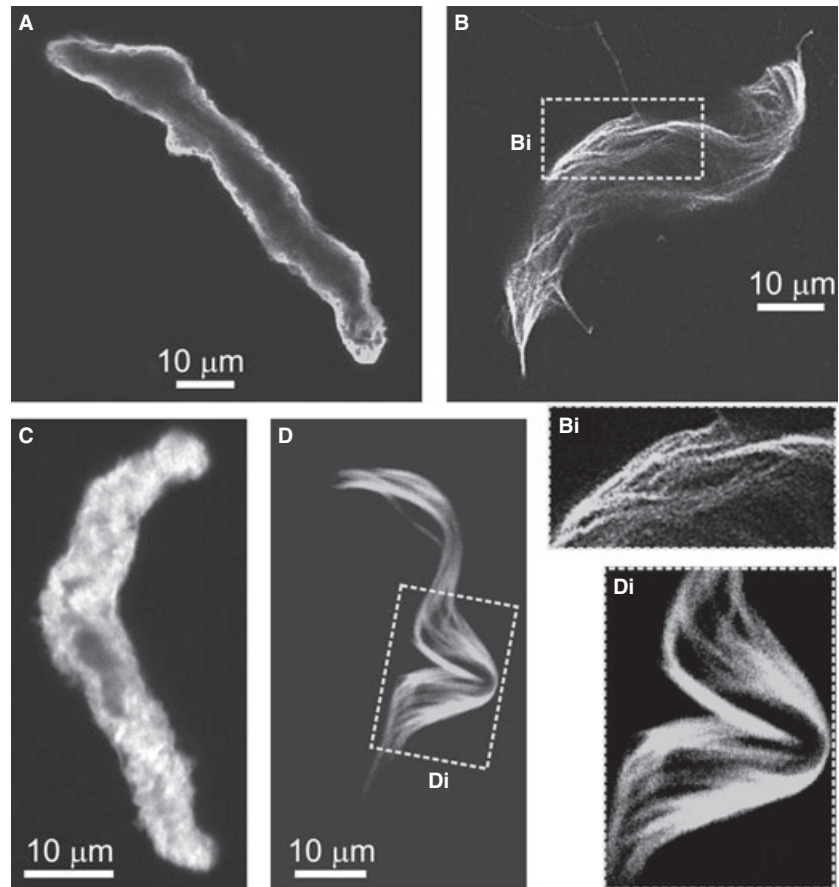
Fig. 7 Immunofluorescent detection of αSM-actin (**A**, **B**) and f-actin (**C**, **D**) in VSMCs (**A**, **C**) and VICs (**B**, **D**). No individual stress fibres were observed in VSMCs where these proteins were densely packed. Note, that higher concentrations of αSM-actin were observed in superficial region of the VSMC (**A**). VICs showed the presence of the stress fibres containing αSM-actin and f-actin. (**Bi**) and (**Di**) show enlarged images of insets in (**B**) and (**D**).

migratory phenotype [14, 15]. Moreover, the absence of stiff cytoskeleton in VICs comparing to VSMCs, even further confirms these implications. Although the speed of growth of filopodia and number of all changes in the shape of VICs decreased with time in isolated VICs, such decrease may be related to an absence of continuous external supplies of cellular nutrients and building components in extracellular solution, and/or the absence of sufficient stimuli to maintain further development of these processes after the maximal adherence of VICs to the bottom of an experimental chamber has occurred. Most importantly, the presence of sprouting VICs in dispersal indicates that these cells may represent phenotypically modulated VSMCs with increased proliferative abilities as sprouting of endothelial cells is associated with their proliferation during angiogenesis and VICs display formation of similar sprouts and formation of new filopodia on such sprouts. Considering the fact that cell migration is sensitive to the proliferative state of VSMCs and non-proliferating cells in culture are not migratory [41, 42] we suspect that VICs have higher proliferative abilities than SMCs and this concept will be investigated in our future studies.

We can hypothesize that in blood vessels in response to certain condition (*e.g.* a disease or tissue injury) the active changes in shape of VICs may lead to the formation of stalk cells by utilizing VIC's filopodia, similarly to the sprouting of endothelial cells during blood vessel formation [14, 43, 44]. Stalk cells, in turn, may form either new VICs or replace apoptotic VSMCs. In this case VICs may play the role of precursors or progenitors of VSMCs in the wall of blood vessels. Although the possible presence of vascular progenitor cells in blood vessels have been extensively discussed (see review [45]) and the presence of potential VSMCs progenitor cells was reported in *tunica adventitia* of aorta of ApoE-deficient mice [46], there is no direct evidence of presence of VSMC- progenitors in the *tunica media* of the blood vessel wall. It was proposed that there may be several distinct types of smooth muscle progenitor cells normally resident in the adult artery wall with the ability to respond to injury or disease-promoting stimuli and differentiate into VSMC-like cells *in vivo* [47]. It is quite possible that VICs can serve as VSMC precursors in the media of the blood vessel.

Using transmitted electron microscopy technique, VICs were previously detected in the *tunica media* of the blood vessel of rodents [7, 8] and this was confirmed in our present study in human arteries. Some of these VICs were detected in close proximity to the elastic membrane of the blood vessel wall. Considering the fact, recent studies confirmed that VSMCs in atherosclerotic plaques are exclusively

derived from the local vessel wall in apoE-deficient mice [48], it is attractive to speculate that sprouting VICs may contribute to neointimal thickening of the vessel wall in certain pathological conditions (*e. g.* during atherosclerosis). The rapid response of VICs, by changing the shape of the cell body and sprouting, to the external stimuli, which in our situation is dispersion of the blood vessel into single cells, allows us also hypothesize that in blood vessel VICs may contribute to the vascular remodelling in response to the vascular injury.

It deserves mentioning, that filopodia also is one of the definitive features of interstitial cells of Cajal in visceral smooth muscle [49–52] as well as in other interstitial-like cells, or telocytes [53] recently described in other tissues such as in uterus [54, 55], pancreas [56], skeletal muscle interstitium [57], epicardium [58] and endocardium [59], bronchioles [60] and parotid glands [61]. As no clear role for filopodia or telopodes [53] in these cells has been determined yet, it is quite possible that these cellular protrusions may serve as an indicator of increased migratory abilities of these cells in different tissues. Notably, recent work by Manole *et al.* implicated that telocytes may be involved in neo-angiogenesis after myocardial infarction [62]. This further suggests that establishing the role of interstitial cells in tissue remodelling during injury is necessary to prove this hypothesis.

In this study we, for the first time, confirmed that VICs are PMVSMCs that are constitutively present in the human blood vessels. The presence of phenotypically modulated VSMCs necessary for maintenance and remodelling of blood vessels under normal physiological conditions has previously been suggested [63] and it is possible that VICs may represent such cells in the vascular wall. To further explore their migratory properties and proliferative properties, the effect of different extracellular media and various factors affecting proliferation and migration of VSMCs on freshly dispersed VICs [64] needs to be studied. Although it was proposed that identification of PMVSMC is particularly challenging, mainly because of an absence of the specific marker for these cells [2], VICs represent a group of cells with multiple features that differ them from contractile VSMCs. We therefore believe that such differences would be reflected at transcriptional and post-transcriptional levels and thus, the comparative studies of multiple genes and protein expression are required as the next step for determining the role of VICs in blood vessels. Such studies may lead either to discovery of the marker of the phenotypic modulation of VSMCs or to the identification of the important molecular pathways controlling mechanisms of the phenotypic modulation in vascular wall. The creation of the pure culture of VICs may also allow studying such mechanisms considering that these would be retained in the culture. VICs therefore may represent a novel model for study of phenotypic modulation of VSMCs during vascular repair, remodelling and disease.

Acknowledgement

Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Paraffin embedded cross-sections of the human resistance gastro-omental arteries stained with haematoxylin and eosin. (**A**, **B**) showing cross-section of the typical arteries used in the experiments from two different patients. The arteries normally displayed between 3 and 6 layers of smooth muscle cells in medial layer and presence of some connective tissue in the adventitial layer. (**Bi**) shows enlarged fragment of the artery depicted by dashed rectangle in (**B**).

Fig. S2. Application of 10 mmol/l caffeine results in contraction of VSMC but not VIC from human gastro-omental artery. Cells were pre-loaded with calcium indicator Fluor-3am. (**A**) transmitted light image. (**B**) fluorescence images of Fluo-3 loaded cells (**a**) before, (**b**) at the moment and (**c**) 10 sec. after 10 mmol/l caffeine application. Red and yellow colours indicate increase in intracellular calcium. Please note the increase in intracellular calcium in response to agonist in both VSMCs and VICs. Please also note the contraction of VSMC (white arrow). Horizontal bar corresponds 20 μm.

Fig. S3. Freshly dispersed VSMC from human gastro-omental arteries does not display any significant changes in shape similar to those recorded in VICs during same period of time. Live imaging performed for 20 min. at room temperature. Transmitted light images taken with 10 min. interval. Horizontal bar corresponds 10 μm.

Fig. S4. Absence of rigid cytoskeleton in VICs compared to VSMCs. (**A**) VIC easily passes into a glass pipette with inner diameter less than half of the size of the cell. (**B**) similar in size VSMC blocks the pipette. Inset (**A1i**) shows that orifice of the pipette (dashed oval) is less than 3 μm. A moderate negative pressure is applied to the pipette. The horizontal bar corresponds 10 μm.

Video S1. Separate collection of isolated VICs and SMCs.

Video S2. Active change in shape of VIC.

Video S3. Absence of rigid cytoskeleton in VIC.

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