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Research Article

The expression levels of three raft-associated molecules in cultivated vascular cells are dependent on culture conditions

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Abstract. Relaying a signal across the plasma membrane requires functional connections between the partner molecules. Membrane microdomains or lipid rafts provide an environment in which such specific interactions can take place. The integrity of these sites is often taken for granted when signalling pathways are investigated in cell culture. However, it is well known that smooth muscle and endothelial cells undergo cytoskeletal rearrangements during monolayer culturing. Likewise affected – and with potentially important consequences for signalling events – is the organization of the plasma membrane. The expression levels of three raft markers were massively upregulated, and raft-associated 5′-nucleotidase activity increased in conventional monolayer cultures as compared with a spheroidal coculture model, shown to promote the differentiation of endothelial cells. Our data point to a shift of raft components in monolayer cultures and demonstrate potential advantages of the spheroid coculture system for investigation of raft-mediated signalling events in endothelial cells.

Key words. Signalling; smooth muscle; endothelial cells; 5′-nucleotidase; annexin.

Introduction

Vasomotor activity is synergistically controlled by all vascular components and involves close cooperation of endothelial and smooth muscle cells [1]. Changes in vessel diameter brought about by smooth muscle cell contraction influence the organization of endothelial plasma membranes. In turn, differences in fluid shear stress, sensed by endothelial cells, are transmitted via biochemical and mechanical pathways to the underlying muscle layer. Since endothelial and smooth muscle cells are readily perpetuated in culture, there exists a wealth of in vitro data pertaining to these signal transduction pathways. Involving both receptors and cytoplasmic proteins, these events originate above all at the plasma membrane. Lipid rafts have been shown to mediate signalling events, and their clustering is believed to stabilize membrane-bound receptor molecules [2]. Raft-mediated signalling is known to play a role in the vasomotor response to nitric oxide (NO) [3].

Annexins are known to be ‘membrane organizers’ and belong to a family of structurally related, anionic lipid-binding proteins [4]. Annexins 2 and 6 both have an affinity for raft-associated lipids [5, 6]; annexin 2 is known to participate in the Ca^{2+}-dependent assemblage of membrane

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rafts on the inner leaflet of the plasma membrane [7]. On the outer leaflet, GPI (glycosyl-phosphatidyl-inositol)-anchored proteins have been identified as readily accessible raft markers, above all in cells of the immune system [8, 9]. 5′-Nucleotidase is one of the GPI-anchored enzymes whose activity is believed to be regulated by the annexin 2-mediated oligomerization of lipid rafts in smooth muscle and endothelial cells [6].

Based on our observation that plasma membrane proteins undergo significant rearrangement during subculturing of cells [10], we searched for a system which combined ease of pharmacological manipulation with a more accurate correlation of the structural parameters we wanted to investigate than conventional monolayer cultures.

Since plasma membrane rafts are known to be centres of signal transduction [2] and their reorganization is likely to affect downstream signal transduction cascades, we compared the expression levels of three known raft markers in endothelial and smooth muscle cells grown under flat (i.e. conventional) and spheroidal culture conditions. Cocultured spheroids spontaneously organize into a smooth muscle core and an endothelial surface layer. This three-dimensional culturing system has been shown to promote the differentiation of endothelial cells and prevent their apoptosis in a smooth muscle-endothelial cell contact-dependent manner [11].

Our investigation demonstrated a massive upregulation of all raft markers investigated in flat cultures of endothelial or smooth muscle cells (single or cocultures) compared with the levels expressed in spheroids and a marked increase in 5′-nucleotidase activity measured in flat as opposed to spheroidal cultures. These findings outline a potentially important methodological problem in the execution and interpretation of studies which are concerned with membrane-associated signalling in vascular cells.

Materials and methods

Cell culturing

Primary cultures of human microvascular endothelial cells and human aortic smooth muscle cells were obtained from Promocell (Heidelberg, Germany) and cultivated according to the distributor's instructions. In initial experiments, primary cultures of myometrial smooth cells were prepared according to the protocol described by Ehler et al. [10]. There was no difference between vascular and visceral smooth muscle cells in our experiments. Densely confluent cultures of cells between passages 2–6 were used throughout. For the assessment of flat cultures, smooth muscle and endothelial cells were grown either separately or together at a seeding ratio of 1:1.

Spheroid cultures (composed exclusively of endothelial or smooth muscle cells or of a mixture of the two types) were prepared according to the protocol described by Korff et al. [11], with one modification: the spheroids were grown individually, in hanging drops, as described for the cultivation of embryoid bodies [12], these being suspended above a Petri dish filled with phosphate-buffered saline (PBS).

Smooth muscle cells, which form the core of the spheroid bodies, are unable to maintain the expression of their distinct protein isoforms under anchorage-independent culture conditions. Smooth muscle-associated proteins (smooth muscle-myosin heavy chain, h-caldesmon) are rapidly downregulated; thus, spheroids were used for experiments within 24 h of cultivation. For immunohistochemistry, spheroids were collected in Eppendorf tubes, pelleted in a 1:1 Tissue Tek:PBS mixture, snap frozen in liquid N_2, and cryostat sections were prepared.

Immunohistochemistry

Smooth muscle biopsies were excised from the human bladder during surgical undertakings that were unrelated to muscle disease. Consent for working with this tissue was obtained from the Bernese Medical-Ethical Commission.

With the aid of a light microscope, they were teased into individual bundles. Ultracryomicrotomy and immunolabelling were performed as described previously [5].

For immunostaining, the cells were seeded onto glass coverslips, fixed at ambient temperature in 4% paraformaldehyde buffered with Na^+-Tyrode's solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose and 10 mM Hepes; pH 7.4) and then permeabilized (for 30 s at ambient temperature) within 0.5% Triton X-100 (in Na^+-Tyrode's solution). Immunolabelling was performed as described by Jostarndt-Fögen et al. [13]. The employed monoclonal antibodies against annexin 6 (purchased from Transduction Laboratories, Lexington, USA) and against annexin 2 [14] have been previously characterized.

Fluorescent labelling was performed using Cy3- (Jackson, Baltimore, USA) or Alexa-conjugated (Molecular Probes, Eugene, USA) secondary antibodies. Negative controls were generated either by absorbing the antibody with purified antigen (for annexin 2 or 6) or by applying a nonbinding primary antibody.

Specimens were examined in a Zeiss Axiophot fluorescent microscope, and images were collected using a digital CCD camera (Ultrapix, Astrocam) or a Bio-Rad MicroRadiance confocal microscope.

5′-Nucleotidase activity

5′-Nucleotidase was assayed using 5′-AMP as a substrate, as described by Babiychuk et al. [5]. Monolayer cultures (10^5 cells/dish) were washed once with Na^+-Ty-

rode's solution containing 2 mM $CaCl_2$ and then incubated in 1 ml of the substrate buffer (Na^+-Tyrode's solution (pH = 8.0) containing 2 mM AMP, 2 mM $MgCl_2$ and 2 mM $CaCl_2$) for 45 min at 37 °C. Spheroids were collected in Eppendorf tubes (2250 cells/spheroid; 30 spheroids per tube), washed and resuspended in 500 µl of substrate buffer, care being taken not to disrupt them during these steps, in order to monitor the activity in the intact complexes. The reaction products were quantified spectrophotometrically (A_{820}) as described by Parkin et al. [15], and the activity was expressed in units of optical density (OD) per 10^4 cells.

Isolation of RNA and synthesis of cDNA

Flat-cultured cells (endothelial cells as well as smooth muscle cells) and spheroids were washed with PBS, resuspended in lysis buffer and lysed by centrifugation through QiaShredder columns (Qiagen).Total RNA was isolated using the RNAeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. Samples were treated on-column with RNAse-free DNAse (30 units) to avoid possible contamination with genomic DNA.

Complementary DNA (cDNA) templates were prepared from 200 ng of total RNA, using the Omniscript RT kit (Qiagen), random hexamer primers (Life Technologies) being employed at a concentration of 2.5 µM in the presence of an RNAseOUT RNAse inhibitor (Life Technologies). Control reactions were performed without RT. The absence of DNA contamination was confirmed by polymerase chain reaction (PCR) using the primer pairs of annexin 2 (see below). A product was obtained only for samples from which cDNA was synthesized following reverse transcription.

Reverse transcription-PCR

PCR was carried out using the PE Biosystems GeneAMP 5700 Sequence Detection System in conjunction with the SYBR Green PCR kits, as recommended by the manufacturer (Applied Biosystems). PCR pimers were designed [using PRIMER EXPRESS software (Applied Biosystems)] to amplify segments containing fewer than 200 bp (in order to maximize efficiency), their specificity being confirmed by measuring the size and purity of the PCR products by 4% NuSieve agarose gel electrophoresis.

Primer sequences were as follows: Human annexin 2 forward primer 5′-TGGATGAGGTCACCATTGTCA-3′ and reverse primer 5′-GGCGAAGGCAATATCCTGTCT-3′, amplifying a 70-bp fragment between nt 201 and 271 of the annexin 2 cDNA; human annexin 6 forward primer 5′-CTTCGGTTGGTGTTCGATGA-3′ and reverse primer 5′-GCTCCCCTCGGATGCTG-3′, amplifying a 70-bp fragment between nt 730 and 800 of the annexin 6 cDNA; human 5′ nucleotidase forward primer 5′-GGCTGCTGTATTGCCCTTTG-3′ and reverse primer 5′-CTGCAGGAACTCTCCAGTGGA-3′, amplifying a 120-bp fragment between nt 1282 and nt 1402 of the human placental 5′ nucleotidase cDNA; 28S ribosomal RNA (rRNA) forward primer 5′-GTTGTTGCCATGGTAATCCTGCTCAGTACG-3′ and reverse primer 5′-TCTGACTTAGAGGCGTTCAGTCATAATCCC-3′, amplifying a 132-bp fragment between nt 4535 and nt 4667 of the human 28S rRNA cDNA; CD31 endothelial cell marker protein forward primer 5′-GACGGTGCAAAATGGGAAGA-3′ and reverse primer 5′-TGACGTGAGAGGTGGTGCTG-3′ amplifying a 67-bp fragment between nucleotides 276 and 343 of the human endothelial cell adhesion molecule CD31 cDNA; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer 5′-CCCCATGTTCGTCATGGGTGT-3′ and reverse primer 5′-TGGTCATGAGTCCTTCCACGATA-3′, amplifying a 99-bp fragment between nucleotides 446 and 545 of the human GAPDH cDNA.

For 30-µl PCR, 2 µl of cDNA template was mixed with 300 nM each of forward and reverse primer and 2 X SYBR Green Master Mix (Applied Biosystems). The reaction mix was incubated at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 1 min at 60 °C. The relative standard curve method was used (ABI Prism 7700 SDS, User Bulletin #2) to translate the cycle number into a relative quantity. The amount of each target was divided by the amount of 28S rRNA to yield a normalized target value. Each gene-specific PCR was performed in triplicate, and data from three independent experiments were analyzed.

Results

Distribution of raft marker proteins

In transverse ultrathin cryosections through bundles of human urinary bladder smooth muscle, capillary endothelial cells are strongly stained for annexin 2, whereas the surrounding smooth muscle ones are only weakly labelled (fig. 1a). For annexin 6 (fig. 1b), the labelling pattern is reversed: smooth muscle cells and pericytes are intensely stained, capillary endothelial cells but faintly so.

No difference in labelling intensity was observed between either raft-associated protein in flat cultures of endothelial and smooth muscle cells. The predominantly cytoplasmic distribution profiles for annexins 2 and 6 were comparable, occasional nuclear labelling being also encountered (fig. 2a–d). The observed pattern of intracellular organization was similar to that already described for fibroblasts and epithelial cells [16–18]. Within smooth muscle cells, a vague but unmistakable codistribution of annexins 2 and 6 with actin stress fibres was apparent (fig. 2b, d).

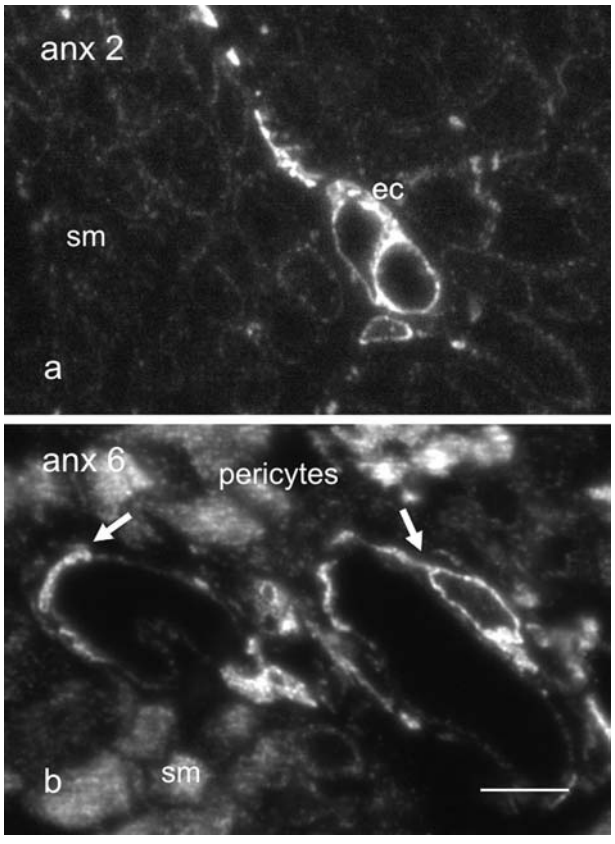


Figure 1. Annexins 2 and 6 are differentially expressed within endothelial and smooth muscle cells in vivo. Transverse ultracryosection through human urinary bladder smooth muscle bundles labelled with antibodies against annexin 2 (*a*) and annexin 6 (*b*). Capillary endothelial cells (ec) stain intensely for annexin 2 (*a*), whereas the surrounding smooth muscle ones (sm) are but faintly immunoreactive. On the other hand, annexin 6 (*b*) abounds within smooth muscle cells and the pericytes (arrows) surrounding capillaries, but it is barely visible within endothelial cells. Bar, 10 µm.

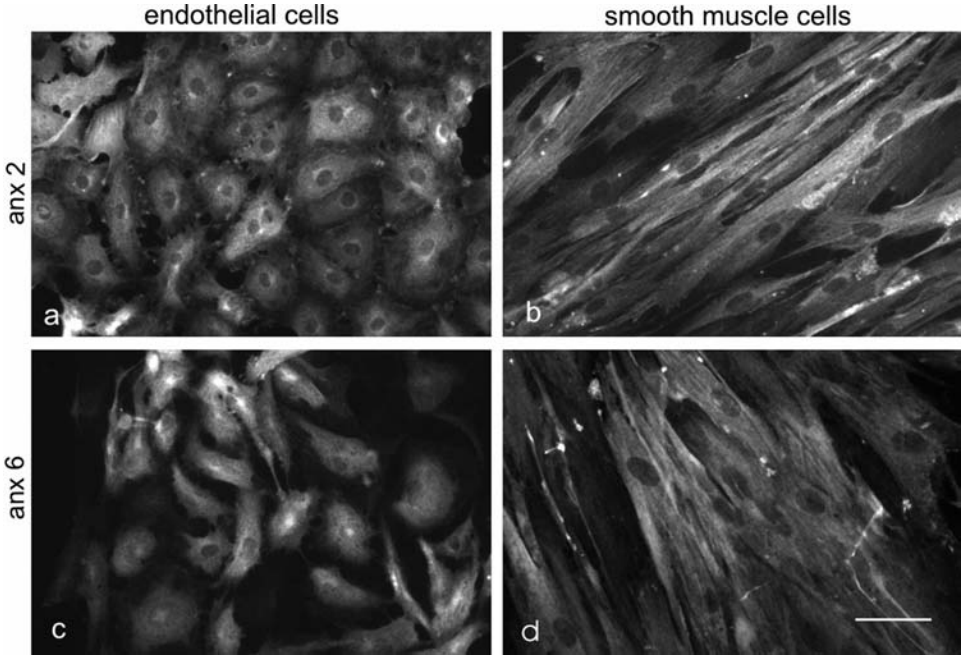


Figure 2. Localization of annexins 2 and 6 in flat cultures of endothelial and smooth muscle cells. Within monolayer cultures of endothelial (*a*, *c*) and smooth muscle cells (*b*, *d*), the predominantly cytoplasmic distribution patterns of annexins 2 (*a*, *b*) and 6 (*c*, *d*) are similar. Nuclear labelling is also encountered occasionally. Within smooth muscle cells (*b*, *d*), the annexins are partially codistributed with actin stress fibres. Bar (*a*–*d*), 10 µm.

We applied a coculturing system which is capable of emulating certain in vivo conditions [11, 19], for the investigation of raft-associated molecules and 5′-nucleotidase reactivity. Endothelial and smooth muscle cells were mixed at a ratio of 1:1 and used for experiments the following day. In spheroidal cultures, endothelial cells become quiescent [11]. Smooth muscle cells are unable to survive if cultivated in suspension for more than 2 days. They do not reduplicate under these conditions. Transverse cryostat sections of endothelial/smooth muscle mixed spheroids demonstrate the almost complete separation between the smooth muscle core and the endothelial surface layer, delineated with an antibody against an endothelial marker [CD31 (fig. 3c)]. A monoclonal antibody against annexin 2 did not distinguish between surface and core of the spheroid (fig. 3a), whereas annexin 6 labelling was largely confined to the smooth muscle cells in the centre of the spheroid (fig. 3b).

5′-Nucleotidase activity

5′-Nucleotidase is considered to be a marker for lipid microdomains, and its level of activity an indicator of raft-mediated signalling events [6]. As such, it was used to gauge the influence of cell-culturing conditions on their signalling properties. In six independent measurements made using the same number of cells derived from flat cultures of either endothelial or smooth muscle cells, 5′-nucleotidase activity was significantly higher than in a like number of corresponding cells derived from spheroidal cultures (fig. 4). In both monolayers and spheroids, the 5′-nucleotidase activity of endothelial cells was consistently lower than that in smooth muscle ones.

Gene expression

The pronounced discrepancy in 5′-nucleotidase activity between the two culture models prompted us to investigate their messenger RNA (mRNA) expression levels of 5′-nucleotidase and of annexins 2 and 6 using quantitative reverse transcription (RT)-PCR (fig. 5).

In accord with our biochemical data (see fig. 4), the expression levels of 5′ nucleotidase were substantially (more than 10-fold) higher in flat cultures of smooth muscle and endothelial cells, than in three-dimensional spheroids composed of the same cell types.

Likewise, in keeping with our biochemical findings, the levels of 5′-nucleotidase mRNA were lower in endothelial cells than in smooth muscle ones. Mixed monolayer cultures and spheroids contained approximately the same mean amounts of mRNA from their contributing cell types (fig. 5a).

The mixed spheroids expressed approximately the same mean amounts of all three gene products as did those consisting of a single cell type only (fig. 5a). In the cocul-

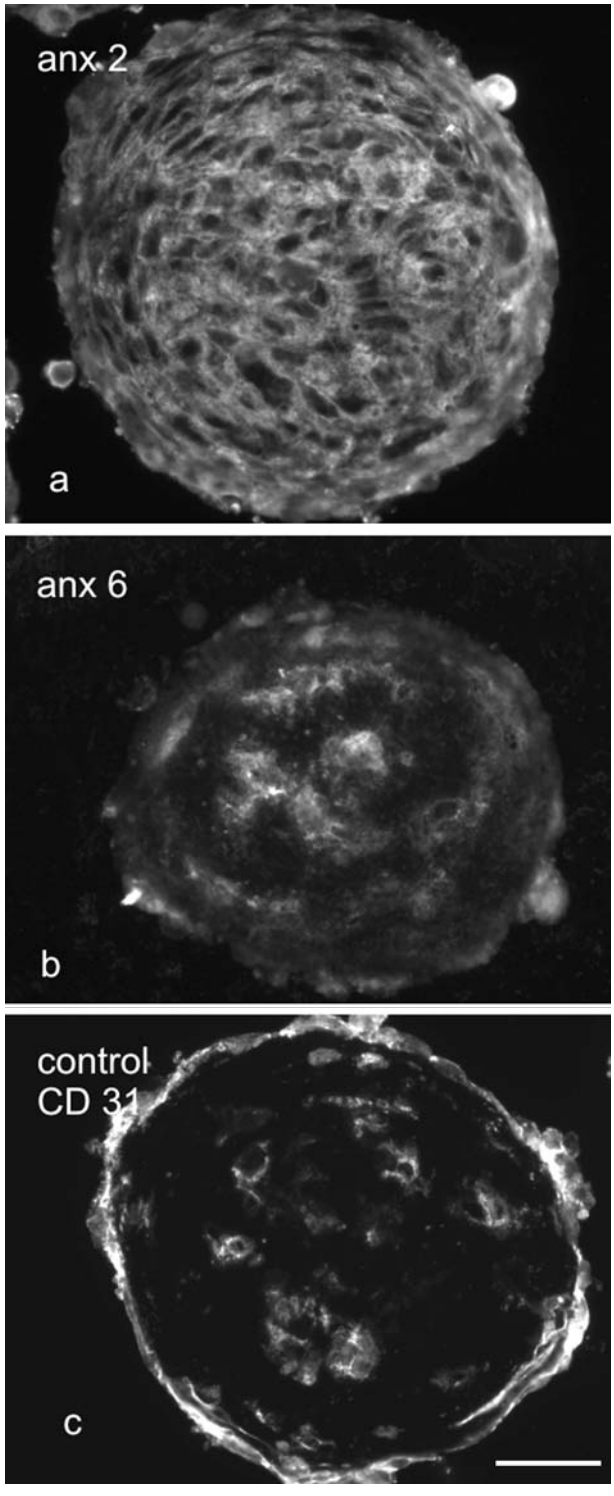
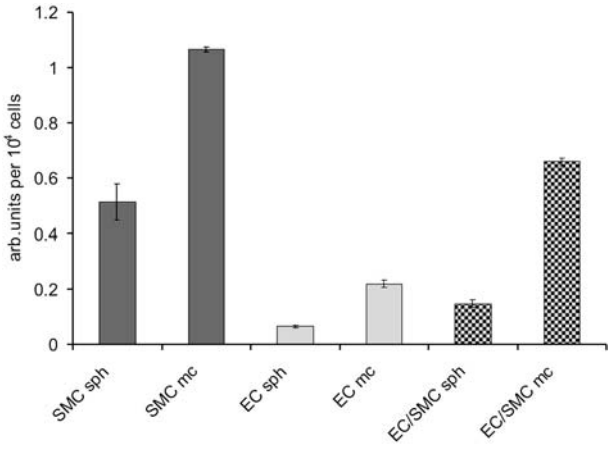


Figure 3. Raft-associated proteins in spheroidal cultures. Transverse cryostat sections of spheroids consisting of endothelial and smooth muscle cells labelled with monoclonal antibodies against annexin 2 (*a*), annexin 6 (*b*) and CD 31 (*c*). Annexin 6 is largely confined to smooth muscle cells at the spheroidal centre (*a*), whereas annexin 2 label does not discriminate between endothelial and smooth muscle cells (*b*). Endothelial cell marker protein CD 31 is predominately found in the peripheral, endothelial layer (*c*). A small number of centrally located, labelled cells demonstrate that the separation of the different cell types is incomplete. Bar, 40 µm.



Figuire 4. 5′-Nucleotidase activity within spheroidal and monolayer cultures. Smooth muscle (SMC) and endothelial cells (EC) were grown in monolayers (mc) or spheroids (sph) either separately or in cocultures (EC/SMC, 1:1). Extracellular 5′-nucleotidase activity was measured in intact spheroids and monolayers as described in 'Materials and methods', using AMP as a substrate. Optical density [(OD) A_{820}] units were calculated per 10^4 cells. Data represent the mean ± SD (bars) of six independent experiments.

tures smooth muscle and endothelial cells were mixed at a ratio of 1:1. In order to confirm that both cell types were present, in addition to the immunofluorescence labelling (fig. 3c), the presence and abundance of the endothelial cell-specific marker protein CD31 was examined by RT-PCR (fig. 5b). The CD31 expression level is reduced by ~50% in the mixed smooth muscle cell/endothelial cell (SMC/EC) cultures compared with the pure endothelial monolayer and spheroid cultures, indicating that the cell ratio did not change as a result of culturing conditions. The overall upregulation of the CD31 expression in the monolayer cultures might be attributed to the more active cell proliferation in the monolayers [20] vs. the cell quiescence in spheroids [11]. On the other hand, the expression levels of GAPDH remained stable irrespective of the cell culture conditions (fig. 5c).

Discussion

Endothelial cells are strategically placed between the bloodstream and the smooth muscle wall. Serving as modulators of smooth muscle contractile responses, they play an important role in the generation and integration of signals. Indeed, they are generally the first cells to be affected by vascular disease. Within a spheroidal coculturing system, which mimics the inside-out assembly of blood vessels, endothelial differentiation and quiescence are believed to be controlled by paracrine mechanisms emanating from the smooth muscle core [11].

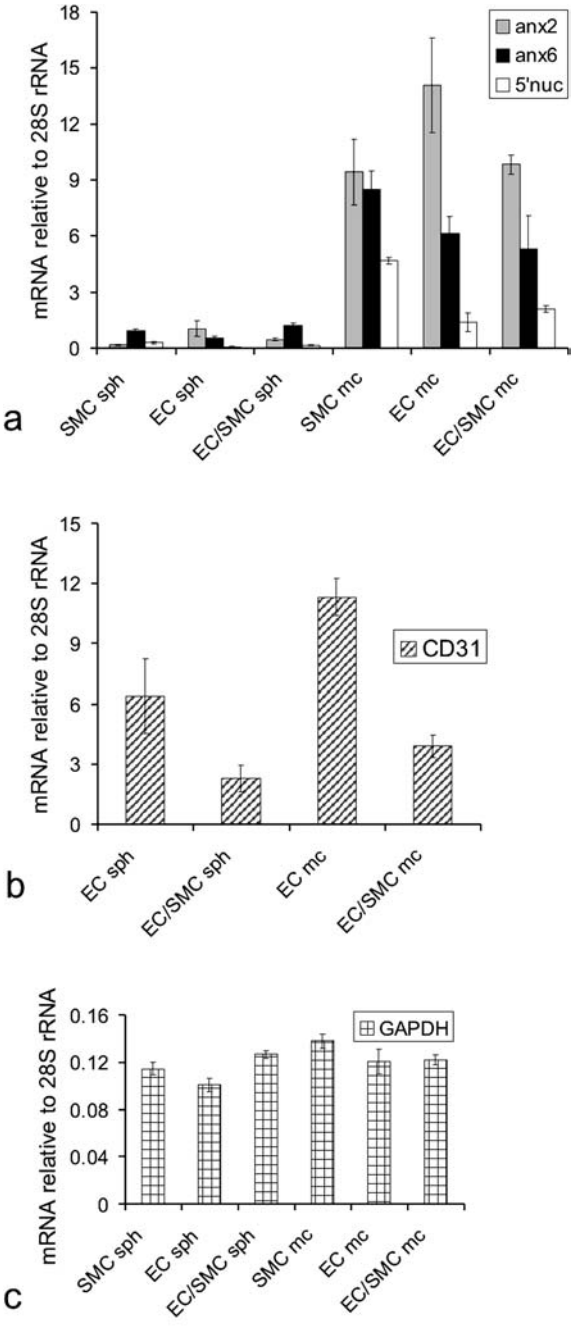


Figure 5. Expression of raft-associated marker genes. Total RNA was isolated from monolayer (mc) and spheroidal (sph) cultures of endothelial (EC) and smooth muscle cells (SMC), and from their cocultures (EC/SMC). The mRNA levels for annexin 2, annexin 6, 5′-nucleotidase (*a*), as well as for CD31 (*b*) and GAPDH (*c*) were determined by quantitative RT-PCR, and the amounts expressed as relative to the 28S rRNA content of each sample. Each column represents the mean ± SD (bars) of three independent experiments performed in triplicate (*a*) or two independent experiments (*b* and *c*). The approximately equal amount of either cell type in the cocultures was determined by measuring the expression level of endothelial cell marker protein CD31 (*b*), which is reduced by 50% in the mixed SMC/EC cultures compared with the individual EC monolayer and spheroid cultures. The expression levels of the raft-associated marker genes in spheroidal cultures were compared to the levels observed in monolayer cultures. The expression level of the housekeeping gene GAPDH remains largely unchanged irrespective of culturing conditions (*c*).

Expression of annexins 2 and 6 in vascular cells

Annexins 2 and 6 both associate with lipid microdomains, the former at a higher affinity than the latter. These lateral assemblies of cholesterol and glycosphingolipids form dynamic structures which are known as lipid rafts or detergent-insoluble glycosphingolipid complexes (DIGs). They are associated with characteristic sets of proteins, which are linked by GPI anchors to the extracellular face. On the inner leaflet they harbour multiple acylated proteins. Membrane rafts can self-associate to form higher-order structures which are physically more stable and constitute the hubs of signalling activity [21, 22]. Recent studies have emphasized a membrane-cytoskeletal connection for annexins: annexin 6 forms a reversible link between the cytoskeleton and the sarcolemma within smooth muscle cells [5], and the F-actin binding site has been recently identified in annexin 2 [23].

Annexins 2 and 6 appear reciprocally expressed – the former predominating in endothelial, and the latter in smooth muscle cells. We observed differential expression levels of mRNA for annexin 2 and annexin 6 in monolayer and spheroidal cultures of the two cell types. These differences were reflected in the immunohistochemical reactions to monospecific antibodies of human urinary bladder smooth muscle and mimicked in the preferential outlining of the endothelial periphery by annexin 6. Otherwise, differences in labelling intensities between annexin 2 and 6 were not observed in cultured cells. It was notable, however, that the intracellular distribution of annexins 2 and 6 in monolayer cultures of both cell types was a predominantly cytoplasmic one, corresponding to a low intracellular [Ca^{2+}] [24].

5′-Nucleotidase activity and expression

5′-Nucleotidase cleaves AMP into adenosine, acting as a potent vasorelaxing agent [25]. Its activity in endothelial cells is equally important, particularly within peripheral regions of the vascular tree where the marked anticoagulant properties of adenosine help to ensure an unrestrained blood flow [26]. As a result of arteriolar and precapillary dilatation, large volumes are redistributed rapidly and hemodynamic parameters are altered while blood is shunted towards another region or a different organ. The effects of 5′-nucleotidase activation are equally important for smooth muscle and endothelium and directed at the lower end of the vasculature.

When comparing the influence of the culture conditions on enzyme activity, we demonstrated a noticeable elevation of 5′-nucleotidase activity in monolayer cultures (fig. 4). The same held true for its mRNA levels; however, the observed upregulation of gene expression was significantly higher (about 10 times in monolayer cultures, fig. 5a). This discrepancy might be attributed to the intracellular accumulation of 5′-nucleotidase and its continuous trafficking between the surface and cytoplasmic membranes, which has been shown in many cell types, including endothelial cells [27–29]. Since we measured the enzyme activity on the cell surface only, a significant proportion of the newly synthesized enzyme has probably escaped detection. It is possible that the changes in the cell membrane architecture brought about by the spheroid culture conditions have a particularly profound effect on the expression levels of the membrane-associated proteins. In addition to the raft associated protein genes, we have also observed upregulation of the mRNA levels of CD31 (fig. 5b). In contrast, the expression of GAPDH was not significantly altered (fig. 5c).

Compartmentalization of signalling

The mRNA levels for both annexins were significantly lower in spheroids than in monolayer cultures, which suggests that the number or size of membrane domains is dependent upon the culture conditions. In smooth muscle cells, the breakdown of regular, ‘contractile’ sarcolemmal architecture [30] during cultivation is well documented [10]. It is thus not surprising that membrane rearrangements can be observed in other cell types and that they extend beyond the microscopically discernible ‘smooth muscle macrodomains’.

It has recently emerged that signalling cascades such as those elicited by insulin [31] or those described for H-Ras [32] require well-defined microenvironments for their correct functioning. And we have shown here that alterations in membrane geometry can elicit significant changes in biological effects. Although the present study was not concerned with the potential influence of such disturbances relayed to downstream effectors within the cytoplasm, our data nonetheless suggest that they are likely to be considerable.

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