Polyubiquitin is a new phenotypic marker of contractile vascular smooth muscle cells

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Abstract

Objective: Medial vascular smooth muscle cells (VSMCs) in healthy vessels are phenotypically distinct from their intimal counterparts in vascular disease. To compare the genes expressed in these phenotypes we have previously performed a differential cDNA library screen on cultured rat VSMCs. The aim of this study was to identify and characterise a 2.8 kb transcript, 2E10, which was highly expressed in freshly dispersed rat aortic VSMCs and downregulated in multiply passaged cultured VSMCs. Methods: Sequence analysis was used to identify the 2.8 kb rat cDNA. After trypsinisation of proliferating cultured rat and human VSMCs, or enzymatic digestion of aortic tunica media, total cytoplasmic RNA was isolated from VSMCs by lysis in Nonidet P-40 and extraction in phenol; 15 μg of total cytoplasmic RNA was used in Northern blot analysis with a 32P-[dCTP]-labelled 2E10 cDNA probe. 35S-[dATP]-labelled 2E10 riboprobe was hybridised in situ to frozen sections of normal and diseased human coronary arteries. Results: DNA sequencing identified 2E10 as a rat polyubiquitin which is homologous to the human polyubiquitin, UbC. Northern blot analysis showed that this polyubiquitin was more highly expressed in differentiated, freshly dispersed rat and human aortic VSMCs compared with their dedifferentiated proliferating counterparts. This also identified a 3.2 kb transcript cross-reacting with the polyubiquitin probe which is specific to differentiated rat VSMCs only. However, expression in growth arrested and proliferating VSMCs was identical, suggesting that UbC does not have a role in VSMC growth arrest. In situ hybridisation of the polyubiquitin riboprobe to sections of diseased human coronary arteries indicated much higher expression in medial than in intimal VSMCs. Northern blot analysis of RNA from the developing rat aorta showed that polyubiquitin expression increased substantially after week 2 of neonatal life, coincident with expression of VSMC-specific contractile proteins. Conclusions: The greater expression of a UbC polyubiquitin transcript in contractile, differentiated VSMCs compared with proliferating, synthetic VSMCs provides a new gene marker for the phenotypic characterisation of VSMCs in vivo. This, and the finding that the developmental induction of expression of polyubiquitin (UbC) mirrors that of VSMC contractile proteins, suggests that ubiquitin, a protein known to associate with and degrade contractile proteins in skeletal muscle, is involved in the function or maintenance of the contractile phenotype of VSMCs.

Keywords: Rat, vascular smooth muscle cells; Human, VSMC; Differentiation marker; Polyubiquitin; Ubiquitin; Contractile function; Gene expression

1. Introduction

In the normal coronary vessel wall, most of the vascular smooth muscle cells (VSMCs) are restricted to the tunica media where they express smooth-muscle-specific contractile proteins and act to maintain vascular tone. However, in many vascular diseases, such as atherosclerosis, restenosis after balloon angioplasty or the obliterator vascular disease associated with chronic rejection of grafted organs, VSMCs accumulate in the intima where they may contribute to the pathogenesis of the vascular lesion. These intimal VSMCs have a lower volume fraction of myofibrils [1] and tend to conform to a synthetic or less differentiated phenotype than their medial counterparts, manifested by a loss of contractile proteins such as α-smooth muscle (SM) myosin and α-SM actin [2].

Recently, Shanahan et al. [3] used differential cDNA library screening to isolate specific gene markers of differ-
entiated, contractile rat VSMCs when compared with proliferating VSMCs in culture. Several of the genes coded for recognised contractile proteins and subsequent in situ hybridisation studies on diseased human coronary arteries confirmed much higher expression of these genes in normal medial VSMCs than in disease-associated intimal VSMCs, confirming the association between gene expression and contractile VSMC function in vivo [4]. The identity of 4 of the differentially expressed genes was unknown, however, their pattern of expression implied a role in VSMC contractile function. Here we report the characterisation of one of these genes, 2E10, a 2.8 kb cDNA clone which was found to be much more highly expressed in freshly dispersed, contractile VSMCs than in proliferating cultured cells. Sequence analysis has identified 2E10 as a polyubiquitin, a polymer protein, which is cleaved into its functional ubiquitin monomers when they are required for specific protein degradation by the cell. Our results indicate that this polyubiquitin is a new phenotypic marker for differentiated VSMCs whose pattern of expression in human vascular tissue and in the developing rat aorta is consistent with a previously unrecognised role for ubiquitin in contractile VSMC function.

2. Methods

2.1. cDNA library construction and differential screening

Poly (A +) RNA was isolated from confluent rat aortic VSMCs which had been cultured for 7 days (D7 VSMCs) using Streptavidin magnetic particles (Promega Corp., Madison, WI) according to the manufacturers instructions. This was used to construct a D7 VSMC cDNA library in λ Zap (Stratagene Inc., La Jolla, CA) using a cDNA cloning kit (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions. Differential screening of 40 000 plaques of the library was carried out as described by Shanahan et al. [3].

2.2. DNA sequence analysis

The 2.8 kb 2E10 cDNA was rescued from the phage cloning vector into Bluescript SK (Stratagene) according to the manufacturer’s instructions. Initially, DNA sequence was generated after the 2.8 kb cDNA was sonicated into 300–400 bp random fragments and cloned into M13p18 [5]. These fragments were sequenced using the dideoxy chain termination method with Sequenase 2.0 (Amersham) using ³²P-[dATP] radiolabel and 6% denaturing acrylamide gel electrophoresis to resolve the sequence. Further sequencing of the 3 prime end of the 2.8 kb cDNA was achieved using 20-base-pair oligonucleotide primers specific to known sequence within the clone. Sequence information was assembled using the Staden sequence assembly program [6]. Nucleic acid and protein sequence alignments were made using the FASTA algorithm with the sequence information of GENBANK.

2.3. Cell culture

Primary cultures of rat aortic VSMCs were established from 12 thoracic and abdominal aortas excised from 12-week-old (adult) Wistar rats. After removal of endothelium and adventitia, enzymatic digestion using collagenase (3 mg/ml) and elastase (1 mg/ml) allowed the isolation of VSMCs from the tunica media for plating or RNA extraction [7]. Twelve rat abdominal and thoracic aortae were harvested on 3 separate occasions for RNA extraction; the RNA from each set of 12 was pooled and used for Northern blot analysis. Enzymatic dispersion of the VSMCs does not cause detectable dedifferentiation or proliferation [8]. Adult human VSMCs were grown from explants of normal aorta obtained from a 32-year-old organ transplantation donor. Cells to be passaged were incubated in trypsin/EDTA solution for 3–4 min, then diluted 1:3 (vol/vol) for rat VSMCs and 1:2 (vol/vol) for human VSMCs, before replating. The cultured VSMCs were incubated at 37°C in 5% CO₂ and given fresh medium (Dulbecco’s modified Eagle’s medium and 20% foetal calf serum) at 48 h intervals. Rat cultures were passaged every 4 days, the human VSMCs every 6 days. Removal of proliferating cultured VSMCs from the cell-cycle (G₀ phase) was achieved by serum deprivation for 48 and 72 h for rat and human VSMCs, respectively. VSMCs were identified by their typical appearance and growth characteristics as well as by positive immunofluorescence with anti-a-smooth-muscle-actin and anti-calponin antibodies. Cultures used in the present study were from the 7th to 12th passages.

2.4. RNA isolation

Isolation of RNA from human medial VSMCs first required enzymatic dispersion of the aortic VSMCs from two separate healthy donor thoracic aorta. The endothelium was removed by scraping the luminal surface of the vessel gently and strips of smooth muscle cells (tunica media) were removed with forceps and chopped into 1–2 mm² pieces. These were digested at 37°C using collagenase (3 mg/ml) and elastase (1 mg/ml) until single cell suspensions were obtained. Freshly dispersed cells and trypsinised cultured cells were collected by centrifugation at 900 rpm. Total cytoplasmic RNA was isolated from VSMCs by lysis in 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM MgCl₂ and 0.5% (vol/vol) Nonidet P-40. Nuclei were pelleted at 3000 rpm for 5 minutes, and the supernatant was made 1.5% with sodium dodecyl sulphate and extracted twice with citrate-buffered phenol (pH 4.5). RNA was precipitated at −20°C overnight in 0.1 vol 3M sodium acetate (pH 5.2) and 2.5 vol 100% ethanol, pelleted by centrifugation at 10000 rpm for 10 min, dried, and resus-
pended in an appropriate volume of diethylpyrocarbonate (DEPC)-treated water.

2.5. Northern analysis

Fifteen micrograms of total cytoplasmic RNA was electrophoresed in 1.5% agarose gels containing 2.2 M formaldehyde in a buffer containing 20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate and 0.5 μg/ml ethidium bromide before being transferred by capillary blotting to nylon membranes (Hybond-N, Amersham). After hybridisation of the probe, filters were washed twice at 65°C for 30 min in 0.1 X SSC/0.1% SDS before exposure to Fuji RX X-ray film for 16 or 24 h.

2.6. In situ hybridisation

Ten fresh coronary arteries, which ranged from normal to diseased, were obtained from recipient hearts at the time of orthotopic transplantation. Samples were mounted in Tissue-Tek OCT embedding compound (Miles Ames Division, Inc., Elkart, IN), snap-frozen in liquid nitrogen and stored at −70°C before sectioning; 8–10 μm sections were cut and mounted onto gelatinised slides, refrozen and stored at −70°C until use. Sections were processed for in situ hybridisation according to Boehm et al. [9]. Hybridisation was carried out using 35S-[aATP]-labelled sense and antisense riboprobes generated by in vitro transcription of full-length 2.8 kb 2E10 cDNA clones from the T3 and T7 promoters of Bluescript SK-linearised plasmids. Slides were exposed to β-max hyperfilm (Amersham) for 3 weeks before being developed, stained with haematoxylin-eosin and mounted.

3. Results

3.1. Sequence analysis of differentially expressed 2.8 kb 2E10 cDNA

A 2.8 kb cDNA clone, 2E10, was isolated from a differential cDNA library screen of differentiated versus proliferating rat VSMCs as described in Section 2. Sequence analysis using random sonicated fragments and primers specific to identified sequence within the cDNA has identified it as a rat polyubiquitin transcript. Fig. 1A shows the 5′ DNA sequence and the predicted amino acid sequence of this polycistronic cDNA, encoding the first ubiquitin repeat and the start of the second. This particular rat polyubiquitin was initially isolated from a rat cerebral cortex and hippocampus λgt10 cDNA library and fully sequenced by Hayashi et al. [10]. The transcript encodes 10 complete head-to-tail repetitive ubiquitin repeats followed by the first 20 amino acids of the previous repetitive ubiquitin and a distinct peptide. Indeed, partial sequencing of the 3′ end of this VSMC transcript has identified the same distinct terminal peptide sequence (Fig. 1B). This particular polyubiquitin appears to be the rat homologue of the human polyubiquitin UbC, and may be referred to as ‘rat UbC’.

3.2. Elevated levels of polyubiquitin (UbC) expression in medial VSMCs

Fig. 2 shows Northern blot analysis of RNA from freshly dispersed rat and human aortic VSMCs compared
Fig. 3. In situ hybridisation of \(^{35}\)S-[dATP]-labelled rat polyubiquitin (UbC) riboprobe to a section of diseased human coronary artery. (A) Haematoxylin-eosin staining of the diseased vessel. (B) Dark-field illumination showing that hybridisation of the antisense riboprobe is high in medial VSMCs but low in intimal VSMCs and adventitial cells. Ad = adventitia; M = media; iel = internal elastic lamina; In = intima composed of VSMCs (identified by immunohistochemistry using α-smooth-muscle-actin antibody).

with their proliferating, cultured counterparts. VSMC RNA from 12 rat aortae was pooled on 3 occasions and used in 4 separate Northern blot experiments. Similarly, two isolates of human aortic RNA were extracted from different healthy aortae and Northern blot analysis was performed separately on each isolate. Rat polyubiquitin (UbC) mRNA expression was higher in the freshly dispersed, differentiated rat VSMCs compared with cultured rat VSMCs and also Rat 2 fibroblasts (Fig. 2A). The Northern blot also identified another larger transcript in the differentiated cells only. This 3.2 kb transcript represents another polyubiquitin transcript, probably with extra ubiquitin repeats, which is widely expressed in various rat tissues [10]. Although expression of this transcript was observed in differentiated rat VSMCs, it was undetectable in the proliferating cultured cells (Fig. 2A).

Fig. 2B shows that, as with the rat VSMCs, human UbC polyubiquitin expression was higher in freshly dispersed than in cultured VSMCs. However, only one transcript was detected in human VSMCs, suggesting that the extra 3.2 kb polyubiquitin transcript identified in rat VSMCs is species-specific.

Because medial VSMCs are essentially quiescent (G_0) and cultured VSMCs proliferating, it was important to eliminate any cell-cycle involvement in the observed changes in polyubiquitin (UbC) gene expression. For this reason, UbC expression was analysed in non-growing (G_0) phase cultured rat and human VSMCs. Northern blot analysis showed no difference in expression in the non-proliferating compared with proliferating cultured VSMCs (data not shown). This suggests that the difference in UbC expression in contractile and synthetic VSMCs is not related to growth arrest.

3.3. In situ hybridisation

To determine whether polyubiquitin (UbC), identified in a cultured rat VSMC cDNA screen, was differentially expressed by human VSMCs in vivo, we performed in situ hybridisation, using a \(^{35}\)S-[dATP]-radiolabelled rat polyubiquitin riboprobe, to sections of 10 normal and diseased human coronary arteries. This demonstrated high expression of polyubiquitin in the medial VSMCs of the vessel with low expression in intimal VSMCs or adventitial fibroblasts (Fig. 3).

3.4. Developmental expression of polyubiquitin (UbC) in rat VSMCs

The expression of rat VSMC contractile proteins has been shown to be progressively upregulated during development from neonate to adult (Shanahan CM, unpublished observations). To investigate the possible role of polyubiquitin (UbC) in the function or maintenance of the contractile phenotype of VSMCs, VSMC RNA was isolated from day 2, week 2, 4, 6, 8 and adult (week 12) rats. GAPDH reprobe is shown to indicate RNA loading. The lower arrow indicates the 2.8 kb UbC transcript; the upper arrow indicates the position of the 3.2 kb transcript which is first identified at week 2.

Fig. 4. Northern blot analysis showing the developmental expression of polyubiquitin (UbC). Rat VSMC RNA was isolated from 2-day-old (D2), 2-week-old (W2), 4-week-old (W4) and adult (Ad) rats. GAPDH reprobe is shown to indicate RNA loading. The lower arrow indicates the 2.8 kb UbC transcript; the upper arrow indicates the position of the 3.2 kb transcript which is first identified at week 2.

4. Discussion

We have shown that a 2.8 kb cDNA (2E10) which was previously identified in a differential screen of cDNA from
cultured rat VSMCs codes for polyubiquitin (UbC). We have also shown that UbC is more highly expressed in freshly dispersed human aortic VSMCs than in their cultured counterparts, and that freshly dispersed rat VSMCs have an extra 3.2 kb UbC transcript, not expressed in the human cells.

Ubiquitin is a highly conserved 76-amino-acid protein which, in a well-characterised ATP-dependent enzymatic process [11, 12] involving 3 accessory enzymes (E1–3), binds to proteins, targeting them for non-lysosomal degradation by the proteasome [13]. Although the signals or mechanisms by which ubiquitin targets proteins for degradation are still unclear, rather more is known about its cellular targets and the variety of cellular processes it regulates which include gene expression [14], cell division [15] and the cellular stress response [16]. There are at least 3 known human polyubiquitin genes, UbA (0.6 kb), UbB (1.2 kb) and UbC (2.6 kb), which differ in their number of ubiquitin head-to-tail repeats; however, the functional significance of each is not known. Polycistronic polyubiquitin mRNA is translated to give a polymer protein of ubiquitin monomers, the number of which depends on the individual polyubiquitin. These covalently-linked ubiquitin monomers are thought to be inactive until, when required by the cell, they are released by an unknown protease which breaks the covalent bonds. The active ubiquitin so released is then free to carry out its specific functions within the cell. It is thought that by transcribing a polycistronic message encoding multiple ubiquitin monomers, the cell saves transcriptional energy when the demand for ubiquitin in the cell is high. Alternatively, polyubiquitins can be seen as ‘stores’ of inactive ubiquitin protein which can be rapidly liberated by simple peptide cleavage as and when required for the removal of unwanted proteins. In our study, attempts to demonstrate polyubiquitin protein in VSMCs were hampered by the lack of a specific antibody for polyubiquitin. Western analysis with an antibody for ubiquitin identified two proteins in differentiated rat VSMCs of the expected size for UbC (data not shown); however, these could equally well represent polyubiquitinated cellular proteins.

Although the role of ubiquitin in vascular smooth muscle is not known, studies in skeletal muscle strongly suggest a role in regulating the turnover of contractile proteins. For example, ubiquitin protein levels are increased in exercise-induced muscle damage [17], consistent with a role for ubiquitin in the degradation of damaged myofibrillar proteins [18]. Also, there is increased ubiquitin proteolysis of skeletal muscle in tumour-bearing rats [19] and during sepsis [20] which is thought to involve the degradation of long-lived myofibrillar proteins since ubiquitinated proteins have been identified in myofibrillar fractions [21].

We found that expression of UbC in vitro correlated closely with expression of genes for contractile proteins: \(\alpha\)-smooth muscle (SM) actin, \(\gamma\)-SM actin, calponin, phos- pholamban, and the smooth-muscle-specific protein SM22\(\alpha\), which are also downregulated in passaged dedifferentiated VSMCs [3]. Also, expression of UbC in vivo was highest in the adult rat aorta. Although commitment to VSMC differentiation occurs early in the developing foetal aorta [22], we have found that substantial expression of contractile proteins occurs only after week 2 of neonatal development (Shanahan CM, unpublished data). Therefore, expression of contractile proteins gradually increases throughout neonatal development with the highest levels being in the differentiated, fully contractile adult cell [23]. Therefore, our results show a clear link between the differentiated, contractile phenotype of VSMCs and elevated levels of UbC mRNA in rat and human VSMCs, suggesting a possible role for UbC in the maintenance or function of the differentiated VSMC phenotype.

Although our results point towards a specific role for ubiquitin in VSMC contraction, ubiquitin has also been reported to be involved in a variety of other cellular processes including cell-cycle regulation, the modification of polypeptide receptors such as the platelet-derived growth factor receptor [24], and the degradation of cellular growth control proteins such as c-jun [25]. It is therefore plausible that ubiquitin may be involved in VSMC processes other than contraction. In particular, elevated UbC levels may have a role in NF-\(\kappa\)B-mediated growth arrest through the degradation of IkB\(\alpha\) [26]. However, we have found no difference in the expression of UbC in quiescent compared with proliferating rat and human VSMCs, implying that UbC is not involved in VSMC growth arrest.

In conclusion, the greater expression of a polyubiquitin transcript in contractile, differentiated VSMCs compared with dedifferentiated VSMCs has provided a new genetic marker for the phenotypic characterisation of VSMCs in the diseased vessel wall. Although the exact role for this gene in differentiated VSMC function remains unknown, our data and observations in skeletal muscle suggest that ubiquitin is involved in contractile function.

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References


