Correspondence

Cloning of genes differentially regulated during change in vascular smooth muscle phenotype

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The arterial vessel wall is composed of a single layer of endothelial cells at the lumen and multiple layers of smooth muscle cells (SMC) forming the media and then the adventitia. The primary function of the medial SMC is to provide the elastic and contractile properties to maintain vascular tone but they can also undergo phenotypic change, as occurs in response to vascular injury and in diseases such as atherosclerosis, to a phenotype where they proliferate, migrate and synthesise more extracellular matrix [1,2]. This phenotypic modulation from the mature 'contractile' state to a less differentiated 'synthetic' state is associated with a loss of cytoplasmic myofilaments and changes in gene expression particularly those encoding cytoskeletal proteins [2,3]. Various workers have identified genes induced in animal models of arterial injury such as the balloon de-endothelialised carotid artery [4] and others have isolated markers of differentiated or proliferating SMC by differential cDNA screening in vitro and identified gene products associated with contractile function or with the extracellular matrix [1]. We have used a primary culture model where the process of SMC phenotypic change occurs spontaneously, without induction with a specific factor, when SMC freshly isolated from the artery wall are plated at less than confluent density [2]. Standard differential display techniques [4] have enabled us to identify genes which show altered expression as cells undergo the process of phenotypic modulation in this primary culture system.

Cultures of SMC were established from thoracic and abdominal aortae of 9–12-week-old rabbits with collagenase and elastase and seeded at sub-confluent density. RNA was extracted from primary cultures using the standard acid guanidinium thiocyanate/phenol chloroform extraction method, as they started to de-differentiate (day 2) and when the change to the de-differentiated phenotype was almost complete (day 4). cDNA was synthesised from the extracted RNA from SMC at these two time points and amplified by PCR using degenerate oligo(dT) primers and arbitrary decamer primers annealed at random positions upstream of the poly(A) tail. Analysis of cDNA fragments on a 6% polyacrylamide sequencing gel showed at least 10 bands differentially expressed. Of the three bands that showed most marked differential expression two transcripts were highly expressed at day 2 while the third transcript showed a marked up-regulation at day 4. These fragments were excised from the gel, re-amplified and cloned and sequenced. Analysis of the sequence of these transcripts showed that the first transcript (accession number AF399638) had 84.2% identity with human tropomyosin-1 (TM-β, accession number M74817), in a 317-bp overlap. The second transcript (accession number AF438159) showed very high homology with human heparan sulphate *N*-deacetylase/*N*-sulfotransferase (NDST, accession number U18918) with 91.2% identity in a 341-bp overlap, and the third transcript (accession number AF408849) had 86.2% identity with the human ribosomal protein gene, rpL17 (accession number T58361), in a 174-bp overlap. In all three cases the homology was at or near the 3′ end of the mRNA. These homologies were confirmed using full-length cDNA clones of rabbit β-tropomyosin (91.7% identity to human TM-β) and rabbit ribosomal protein rpL17 (88.2% identity to human rpL17) obtained from a rabbit SMC cDNA library prepared in our laboratory from aortic SMC in culture. RT-PCR performed with specific primers for these transcripts using cDNA synthesised from the RNA extracted from SMC primary culture at day 2 and 4 confirmed the differential expression of these transcripts (Fig. 1a).

Antibodies against NDST and tropomyosin allowed us to assess the relevance of these findings by examining the expression of these proteins in vivo after balloon catheter de-endothelialisation injury to the rabbit aorta [5]. Immunoblotting of protein extracts with the tropomyosin antibody showed the presence of two bands at approximately 39 kDa and 36 kDa (Fig. 1b), which likely correspond to smooth muscle β (TM-1) and α isoforms respectively. Higher expression of the 39-kDa isoform than the 36-kDa isoform was observed with some decrease at 7 days after injury. The 36-kDa isoform showed more variation with lower levels most evident at 1 and 7 days after injury. The NDST protein band (at the expected size of 97 kDa) was highly expressed in normal (uninjured) artery, and also showed lower levels at 1 and 7 days after injury (Fig. 1b). We have shown previously a change in SMC phenotype quantitated by a decrease in volume fraction of myofilaments within the same time frames by cells both in vitro [2] and in vivo following arterial injury [5].

Decreased overall levels of tropomyosin along with other cytoskeletal proteins have previously been suggested in SMC of a more 'synthetic phenotype' [3]. Two main tropomyosin isoforms are expressed in smooth muscle, identified as TM-1 or smooth muscle β and smooth muscle α, which are thought to play a role in stabilisation of actin filaments in this cell type. Down-regulation of TM-β tropomyosin expression has been reported in dedifferentiating chicken gizzard and vascular SMC in culture, while smooth muscle TM-α converted to TM-F1-α and TM-F2-α [3]. Our results for TM-β mRNA suggest a similar trend, with expression high at day 2 of primary culture but decreased to very low levels at day 4. In addition our in vivo observations point to varied regulation of the TM-α and -β isoforms with some increase in TM-β up to day 3 followed by a decrease. This suggests for the first time, in both the injured artery and primary culture models, that expression of this tropomyosin isoform is transiently up-regulated as SMC begin the process of phenotypic modulation, as well as being decreased to low levels once cells have modulated to the 'synthetic' phenotype.

Our previous work has suggested that there is a constant turnover of heparan sulphate in contractile SMC and that this turnover (synthesis and degradation) may be essential for

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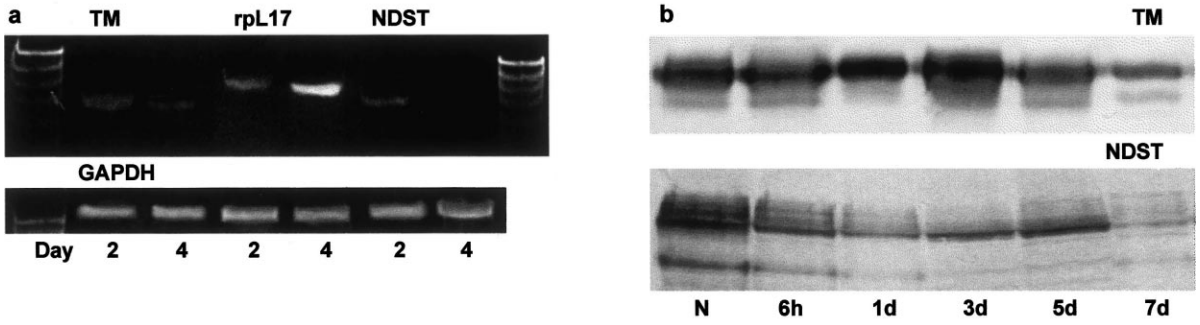

Fig. 1. a: RT-PCR of transcripts at day 2 versus day 4 of SMC culture using specific primers from the sequence analysis of the clones. Lanes 1 and 8 are molecular weight markers (Bluescript digested with *Hpa*II). Lanes 2 and 3 are tropomyosin at day 2 and day 4 SMC culture respectively (using specific forward primer 5′-GACTCAGACCGCAAATAC-3′, and reverse primer 5′-TGAAATCTCCACGTTCTC-3′); lanes 4 and 5 are rpL17 at day 2 and day 4 (forward primer, 5′-TCAAGATGTCGAAGCGAGG-3′ and reverse primer, 5′-GGAGAATCATG-CAATGCTG-3′); and lanes 6 and 7 are NDST at day 2 and day 4 (forward primer, 5′-TATCCACTCCATCCTCAAG-3′ and reverse primer, 5′-AGGGTATAAACTCTGGCAC-3′). RT-PCR-amplified fragments (707 bp) of the rabbit GAPDH gene cloned in our laboratory are shown as controls (forward primer, 5′-CTTCACCACCATGGAGAAGG-3′ and reverse primer, 5′-CTTACTCCTTGGAGGCCAT-3′) using the same RNA samples at day 2 and 4. b: Western blot analysis of tropomyosin (antibody TM311; Sigma) and NDST (polyclonal antibody generated against a human NDST peptide; gift from Dr J Stow, University of Queensland) expression in vivo. Lane 1 is normal artery extract; lanes 2–6 are artery extract at 6 h, 1 day, 3 days, 5 days, and 7 days after injury. Two bands of tropomyosin expression are seen at 39 and 36 kDa. NDST is seen as a 97-kDa band.

maintenance of normal phenotype [5]. Certainly the normal 'contractile' cell phenotype can be maintained in vitro by the addition of heparin or heparan sulphate and the addition of heparanase results in a change towards the synthetic phenotype. Addition of arterial heparan sulphate to injured arteries also inhibits the development of neointimal thickening after arterial injury. Thus it is not surprising that NDST, a key enzyme of heparan sulphate synthesis, may also be part of the process of change in cell phenotype. Our results show that NDST is expressed by most cells in the normal artery wall indicating constant turnover of heparan sulphate, but its expression is rapidly down-regulated after arterial injury. Indeed it is possible that change in the sulphated epitope of heparan sulphate around SMC, by change in the levels of NDST, may be just as important as decreased overall levels of heparan sulphate since it is known that changes in sulphated domains are critical to functional interaction of heparan sulphate in other systems.

The up-regulation of rpL17 expression by SMC at day 4 in primary culture suggests a role for ribosomal up-regulation in the latter phase of the process of SMC modulation from a differentiated 'contractile' to a less differentiated 'synthetic' phenotype in which the cells synthesise much more protein and proliferate in response to mitogens. A recent differential display study of genes expressed 7 days after injury to the rat carotid artery showed increased expression of a ribosomal phosphoprotein [4], consistent with this premise. The levels of mRNA for ribosomal proteins appear to be coordinately regulated and depend on the cell's need for protein biosynthetic capacity. Changes in the state of cellular differentiation or de-differentiation will lead to a decrease or increase in protein synthesis in the ribosome such that ribosomal proteins are generally decreased with cell differentiation and increased with cell de-differentiation. For example a number of ribosomal protein genes are down-regulated during differentiation and up-regulated during de-differentiation of myoblasts and ribosomal protein genes are up-regulated in tumours and cell lines. Thus expression of rpL17 in our system is consistent with increased protein synthesis as cells modulate their phenotype.

In summary, we have identified three genes differentially expressed in primary culture of vascular SMC that are associated with the process of change in SMC phenotype seen under these conditions and corresponding changes in vivo after vascular injury. Since a similar change in phenotype is seen in vascular diseases such as atherosclerosis [1,2], these genes may play a role in vascular pathology.

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