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Smoothelin Expression Characteristics: Development of a Smooth Muscle Cell *in vitro* System and Identification of a Vascular Variant

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ABSTRACT. Recently we described a protein, smoothelin, that has been exclusively found in smooth muscle cells (SMC). The human cDNA has been cloned from a colon cDNA library and the putative protein sequence was deduced. Smoothelin does not belong to a known protein family but shows a partial homology with members of the spectrin family. Transfection studies revealed that smoothelin has an affinity for actin and is either capable of forming filamentous structures or colocalizes with such structures. The protein is expressed in visceral as well as vascular tissues of all vertebrate classes. A study on the distribution of smoothelin in the vascular and placental system showed that smoothelin expression was largely restricted to the muscular pulsating blood vessels. Therefore, we hypothesized that smoothelin is expressed in contractile SMC only (36, 37).

No expression of smoothelin was observed in established cell lines of SMC. In tissue explants smoothelin mRNA concentration decreases to undetectable levels within 12 hours after dissection as was in general the case in primary cell cultures. Here we report on continued smoothelin expression for several passages observed in a human prostate primary cell culture system. Smoothelin was demonstrated to colocalize with actin stress fibers but not with desmin filaments. This culture system offers opportunities to study the cytological localization of smoothelin, interactions with other proteins and should provide a system to test the promoter of the smoothelin gene. On immunoblots the molecular weight of smoothelin differed between visceral and vascular smooth muscle tissue with apparent molecular weights of respectively 59 kDa and 94 kDa. There is no evidence for the existence of another gene coding for the 94 kDa smoothelin. Thus, posttranslational modification, alternative splicing and dual promoter control are the alternatives for the expression of two isoforms of smoothelin.

Smooth muscle cells (SMC) are found in those organs that are involved in basic physiological functions such as breathing, reproduction and transport of nutrients and oxygen. Although SMC appear more 'primitive' in organization and differentiation, as compared to striated (cardiac and skeletal) muscle cells, they developed simultaneously during evolution (13, 23). Skeletal and cardiac muscle are referred to as striated muscle because of their conspicuous transverse striations showing the high level of organization of actin and myosin filaments in the myofibers. On the other hand, in SMC actin and myosin filaments have a less ordered organization that has still to be clearly defined. Both striated muscle cell types have a terminal differentiation, i.e. once they become a skeletal or a heart muscle cell they will, under physiological conditions, not return to a previous differentiation stage. In contrary, SMC can shut-

tle between a contractile and a synthetic/proliferative phenotype, a process called modulation (4, 5). During embryogenesis striated muscle cells differentiate and migrate according to well defined developmental pathways whereas the embryological origin of smooth muscle cells appears to be diverse and is still a matter of debate (15, 16). SMC aligning the digestive and genital tracts appear to be derived from the mesodermal plate whereas SMC of the vascular system are suggested to be recruited from the surroundings of the blood vessels and to differentiate into SMC under the influence of factors secreted by endothelial cells (38).

One reason for the relatively poor understanding of SMC biology is the lack of markers indicative for the phenotypes of these cells and/or for their embryological origin. A number of proteins, such as calponin, metavinculin and α -smooth muscle actin, have been suggested as markers for SMC (7, 10, 11, 12, 14, 19, 31, 33). However, they are also found in other tissues during embryonic development and in certain pathological conditions (1, 2, 3, 22, 25, 34, 35). The protein that can be qualified as most SMC specific is the smooth muscle

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myosin heavy chain isoforms SM1 and SM2 (21, 26). These proteins are found only in SMC and are expressed relatively late during ontogenesis. As stated by Owens (26) "It is critical to distinguish proteins that are characteristic of a given stage (or state) of SMC differentiation/maturation versus proteins that alone can serve as definitive markers for identification of SMC lineages to the exclusion of all other cell types". Recently, we described the detection and characterization of a smooth muscle specific protein, smoothelin (36, 37). We hypothesized that the protein might be specific for the contractile phenotype of SMC. This hypothesis was based on four characteristics of smoothelin.

1) The absence of the protein in cultured SMC. No smoothelin was found in the SMC lines and the primary cultures of SMC tested. This might be due to selection (only proliferative SMC will continue to grow in culture) or to dedifferentiation (contractile SMC revert to the proliferative SMC).

2) The decline of smoothelin transcription in tissue explants. It was found that smoothelin mRNA disappears within 12 hours after dissection of the smooth muscle tissue (36).

3) The distribution of smoothelin in the vascular system. The smoothelin content of a variety of blood vessels in adults and during vasculogenesis of the placenta was investigated by immunofluorescence and immunoblotting. Smoothelin concentrations paralleled the contractility of the vessels (37).

4) The affinity of smoothelin for actin. Since actin is one of the major components of the contractile machinery, the affinity of smoothelin might indicate a function in the contractile apparatus of SMC.

These data suggested that smoothelin is a marker for SMC of the contractile type and as such for the final differentiation stage of SMC.

In this paper we summarize the present knowledge about smoothelin and demonstrate its suitability as a late differentiation marker, i.e. a marker for SMC of the contractile phenotype. A cell culture system is presented which maintains smoothelin synthesis. In addition, we show that a vascular-specific form of smoothelin occurs. The existence of the two isoforms may provide some insight in the differences between vascular and visceral SMC.

MATERIALS AND METHODS

Tissue samples. Normal adult human tissues, obtained at autopsy, were immediately frozen in liquid nitrogen and stored at -80°C until use. Tissues from animals (pig, dog, rabbit, rat, mouse) were dissected within a few minutes and immediately frozen in liquid nitrogen. Smooth muscle cells from human vena, human uterine and mammary artery, human prostate and neonatal chicken gizzard were obtained

by enzymatic dispersion (collagenase/pancreatin: Life Technologies, Gaithersburg, MD, USA). For immunohistochemistry tissues were mounted in Tissue-Tek (OCT-compound; Miles Inc. Elkhart, IN, USA), and 3 to 5 μm thick sections were cut at -25°C and air-dried overnight at room temperature or immediately fixed with methanol (at -20°C for 5 min) followed by acetone (-20°C for 30 sec) and airdried for 3 hours before use. Cultured cells were grown on microscopic cover slips and fixed by shortly dipping in methanol/acetone at -20°C .

Culture of prostate epithelial cells. Fresh transurethraly resected prostatic tissue from benign prostate hyperplasia patients was cut into 1–2 mm^3 pieces and spread in a 25 cm^2 flask. Fragments were allowed to adhere to the plastic substrate and covered with 2 ml of 'collection medium' [Dulbecco's Modified Essential Medium supplemented with 20% fetal calf serum, penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and amphotericin (2.5 $\mu\text{g}/\text{ml}$), all purchased from Life Technologies]. Explants were allowed to settle for at least 96 hours, then medium was changed twice a week. When cells started to migrate out of the tissue fragments 'collection medium' was replaced by 'smooth muscle specific medium' [MDCB-131 medium supplemented with 15% horse serum, 10 mM HEPES pH 7.2, 2% penicillin/streptomycin, 2% MEM-Eagle's solution of non-essential amino acids (all Life Technologies, Gaithersburg, MD), 5 $\mu\text{g}/\text{ml}$ insulin, 10 $\mu\text{g}/\text{ml}$ transferrin, 5 ng/ml sodium selenite, 0.1 μM estradiol, 0.1 μg dexamethasone (all from Sigma, St. Louis, MO)]. The 'smooth muscle specific medium' was mixed with conditioned medium from cell line LNCAP at a ratio 3:1. The conditioned LNCAP medium was obtained by growing the cells in base medium (MDCB-131, 15% horse serum, 10 mM HEPES pH 7.2, 2% MEM Eagle's solution of non-essential amino acids) for 3–5 days. Medium was separated from the cells by filtration and was stored at 4°C until use. Prostatic cell cultures were maintained in an atmosphere of 5% $\text{CO}_2/95\%$ humidified air. Every 2–3 days the medium was replaced. Once the cells had reached confluency they were trypsinized and transferred to fresh flasks in a 1:5 dilution.

Antibodies. Antibodies used in this study were:

1. The mouse monoclonal antibody R4A directed against smoothelin (36).
2. Polyclonal rabbit antiserum (pDes) to chicken gizzard desmin (27).
3. Monoclonal antibody sm-1 to smooth muscle actin was purchased from Sigma Immuno Chemicals (St. Louis, MO, USA) (31).
4. Affinity purified rabbit antiserum (pKer), directed against keratin and purchased from Euro-Diagnostics (Apeldoorn, The Netherlands) (28).

In addition, rhodamine-labeled phalloidin (purchased from Molecular Probes Inc. Eugene, OR, USA) was used to stain actin stress-fibers.

Immunohistochemistry. For immunofluorescence 3 to 5 μm thick cryostat sections were pretreated for 5 min with

0.5% Triton X-100 (BDH Chemicals Ltd., Poole, UK) in phosphate buffered saline (PBS: 137 mM NaCl, 13 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 3 mM KH_2PO_4 pH 7.4; Merck, Darmstadt, Germany), followed by a PBS washing step for 5 min. Methanol/acetone fixed sections and fixed cells were used without Triton X-100-pretreatment.

Fixed cells or tissue sections were incubated with the primary antibody for 30 min at room temperature, washed with PBS and incubated with a secondary antibody conjugated to either fluorescein isothiocyanate (FITC) (goat anti-mouse-IgG-FITC, Southern Biotechnology Associates Inc., Birmingham, AL, USA), or Texas Red (TR) (goat anti-rabbit-Ig-TR/goat anti-mouse-IgG1-TR; SBA), or horseradish peroxidase (peroxidase-conjugated rabbit anti-mouse; DAKO A/S, Copenhagen, Denmark). Incubations with secondary antibodies were performed for 30 min at room temperature. Peroxidase activity was demonstrated with 4-amino-9-ethylcarbazole (AEC; Aldrich Chemical Company, St. Louis, MO, USA). After three washing steps with PBS, the fluorescently stained tissues were mounted in Mowiol (Hoechst, Frankfurt, FRG) or, for AEC stained sections, in Kaiser's glycerin-gelatin (Merck).

Protein gel electrophoresis and Western blotting. Approximately 40 cryostat sections (each 20 μm thick) of fresh frozen tissues or about 10^6 cultured cells were collected, washed with 1 ml PBS and centrifuged for 5 min at $12,000 \times g$. After centrifugation the pellet was subjected to a Triton X-100 extraction step and after a PBS wash suspended in 1% Triton X-100, 5 mM ethylene-diamino-tetra-acetic acid disodium salt dihydrate (EDTA; Merck), 0.4 mM phenylmethylsulfonyl fluoride (PMSF; Merck) in PBS, pH 7.4 (36), and extracted for 5 min on ice. After centrifugation for 5 min at $12,000 \times g$, the pellet was washed in 1 ml PBS. After a final centrifugation step (5 min, $12,000 \times g$), the cytoskeletal preparation was dissolved by boiling for 4 min in sample buffer (18), containing 2.3% sodium dodecylsulfate (SDS) and 5% β -mercaptoethanol (Bio-Rad Laboratories, Richmond, CA, USA).

For one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini Protean II Electrophoresis Cell (Bio-Rad Laboratories) 7.5–10% polyacrylamide slab gels containing 0.1% SDS (18) were used. After electrophoretic separation, the proteins were stained with Page Blue 83 (BDH Chemicals Ltd.) or subjected to Western blotting. Culture supernatant of monoclonal antibody R4A directed against smoothelin was used in a 1:5 dilution and immunodetection with horseradish peroxidase conjugated rabbit anti-mouse-Ig (DAKO A/S) was performed according to standard procedures.

Southern blot analysis. Total nuclear human DNA was isolated from peripheral white blood cells by phenol extraction according to standard procedures (30). DNA was restric-

tion digested and transferred to HiBond (Amersham, UK) (32). Filters were hybridized with 50–200 ng of smoothelin cDNA probe labeled with [α - ^{32}P]dATP by random priming (9) at a temperature of 60°C (6). Filters were washed at a final stringency of 0.1 SSC/0.1% SDS at 60°C.

In situ hybridization. The probe used for chromosome 22 identification has been described by Kurahashi and coworkers (17). Probe labeling and fluorescent in situ hybridizations (FISH) was carried out as described previously (8). FISH with probe cHKAD-25, specific for chromosome region 22q23-22qter (17) were performed simultaneously with a smoothelin cosmid probe.

RESULTS

Characteristics of the two isoforms of smoothelin. Sections of different smooth muscle tissues from a number of species were incubated with antibody R4A. SMC of the visceral tissues, the urogenital tract and the vascular system reacted all with monoclonal antibody R4A in immunofluorescence microscopy (Fig. 1). In all these tissues R4A positive SMC displayed a strong cytoplasmic staining. However, in immunoblotting a clear difference was revealed between visceral/urogenital tissues on the one hand and vascular tissue on the other. The visceral and urogenital tissues contain a smoothelin protein of 59 kDa. The main reactive protein in vascular tissue showed as a 94 kDa band in human, pig, rabbit and dog. In addition, in these tissues a faint band was observed at 59 kDa (Fig. 2). The 'heavy' isoform of smoothelin is unlikely to be due to epitope-sharing of two unrelated molecules. Recently, we generated two new monoclonal antibodies against recombinant smoothelin. The epitopes recognized by these monoclonal anti-bodies differed from the one reacting with R4A. However, they still recognized both isoforms (unpublished results).

The two forms of smoothelin are not transcripts of two different genes. In situ hybridization on metaphase spreads showed that only hybridizing loci are found on both chromosomes 22 (Fig. 3). Southern blotting of genomic human DNA digested with a variety of endonucleases and hybridized with the full size (for the visceral form) human cDNA of smoothelin, showed banding patterns as expected for a single copy gene (Fig. 3).

Localization of smoothelin. The localization of smoothelin was investigated by immunohistology and immunofluorescence microscopy. Previously, studies on human tissue sections showed that in tissues no colocalization could be demonstrated of smoothelin and

Fig. 1. Double staining immunofluorescence microscopy of a small human muscular artery (a, b), human duodenum (c, d), human bladder (e, f) and human arector pilli muscle (g, h). Sections were incubated with smoothelin monoclonal antibody R4A (a, c, e, g), rabbit desmin antiserum (b, h) and rabbit keratin antiserum (d, f). Smoothelin and desmin display similar but not identical staining patterns, whereas smoothelin and keratin are expressed in different tissues.

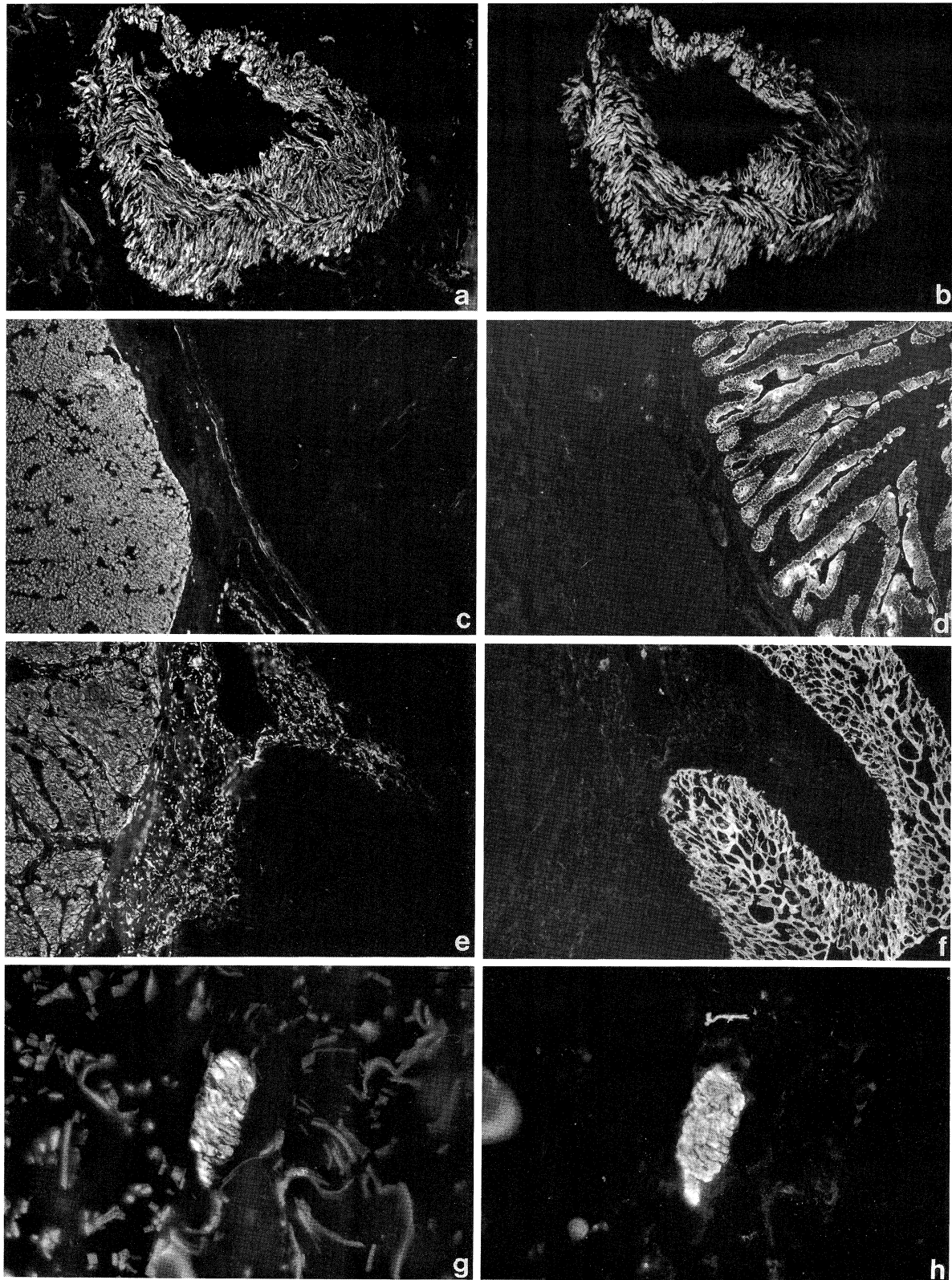


Fig. 1.

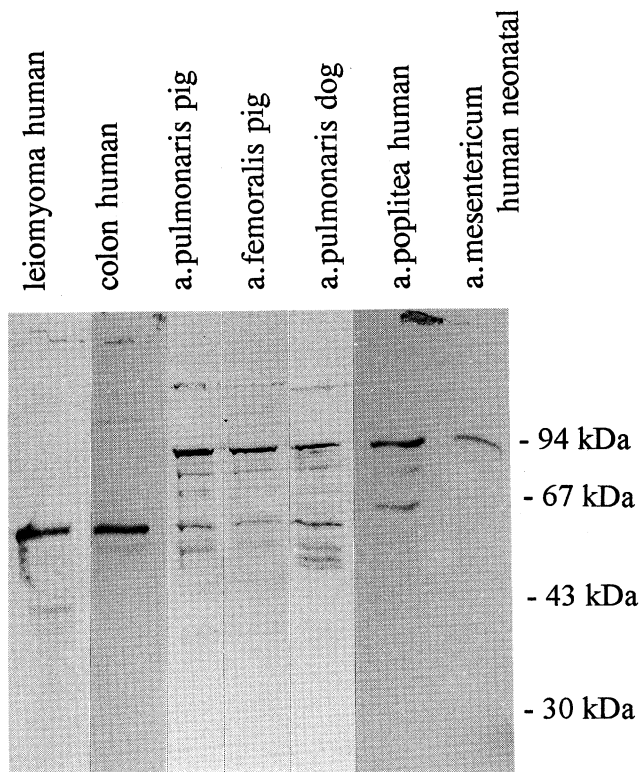


Fig. 2. Immunoblotting of different tissues of several species. The visceral tissues display a clear band at 59 kDa whereas the main band for all the vascular tissues is at 94 kDa. With exception of the neonatal tissue all arteries also contain a little of the 59 kDa protein and display additional bands, likely due to some degradation of the tissue.

other SMC components such as desmin, vimentin and tubulin (36). Primary cultures of SMC of several human tissues, such as colon, arteries and uterus lost expression of smoothelin almost directly after culturing. Whether this was due to an immediate blockage of smoothelin gene transcription or selection of proliferative SMC during the initial phase of culturing was unclear. However, presence of smoothelin was demonstrated to be maintained in primary cell culture of cells derived from a prostate epithelial cells. By immunohistochemical staining it was demonstrated that the cells contained desmin, smoothelin, vimentin and α -smooth muscle actin (Fig. 4). The presence of the smoothelin and desmin protein could be demonstrated for four passages. The concentration of smoothelin and desmin was highest in the first passage and decreased gradually during subsequent passages. Also the number of smoothelin positive cells decreased during the following passages. No changes were observed in the expression of α -smooth muscle actin and vimentin which were also found in later (>5) passages (data not shown). Smoothelin appeared to colocalize with or be attached to actin stress fibers. Smoothelin synthesis appeared to be main-

tained in cells with a slender, spindle-like configuration, but was lost in cells showing a more rounded or square shape (Fig. 4).

DISCUSSION

Smooth muscle cells differ from striated, skeletal and heart, muscle cells in structure, molecular composition and contractile characteristics. The contractile apparatus of SMC is differently organized. The contractile fibrils do not exhibit a sarcomeric repetitive structure, as in striated muscle cells. In conjunction with this difference a number of structural or associated proteins involved in the architecture of striated myofibrils, such as titin and nebulin, are not found in SMC, whereas other proteins such as metavinculin, SM22 and calponin, are almost exclusively found in SMC (10, 11, 12). It is expected that the differences in the structural organization of SMC (versus striated cells) will be reflected in differences of the proteins used for its construction. Although a number of SMC specific proteins have been identified and characterized, so far smoothelin and smooth muscle myosin heavy chain SM1 and SM2 appear to be the only proteins, exclusively expressed in (contractile) SMC (21, 36, 37).

One of the obstructions in studying the localization and function of smoothelin and its interaction with other SMC components has been the lack of a cell culture system in which smoothelin expression was maintained. Only primary cell cultures from embryonic chicken gizzard were occasionally observed to continue smoothelin synthesis during the first passage (36). The availability of primary cell cultures of cells derived from human benign prostate hyperplasia that reproducibly continue to synthesize smoothelin for a number of passages will accelerate the investigation into the localization and the dynamics of interaction with other proteins, and eventually will help to elucidate the function of smoothelin. The data presented here provide an example of the opportunities this system may provide. The clear cytological colocalization of smoothelin with actin in these human cells confirms the results previously obtained with primary cell cultures from embryonic chicken gizzard (36). However, the prostate system can also be used to study the fading of smoothelin in SMC over a number of passages and its relation with contractility and organization of the contractile apparatus. In addition, this system provides opportunities to test promoter-deletion constructs of the smoothelin gene.

The full implications of the occurrence of a second (iso)form of smoothelin, that seems to be specific for vascular SMC are not clear yet. In the view of recent publications on SM22, dealing with distinct transcriptional regulatory programs in vascular and visceral SMC, this is an intriguing observation (20, 24). Here we

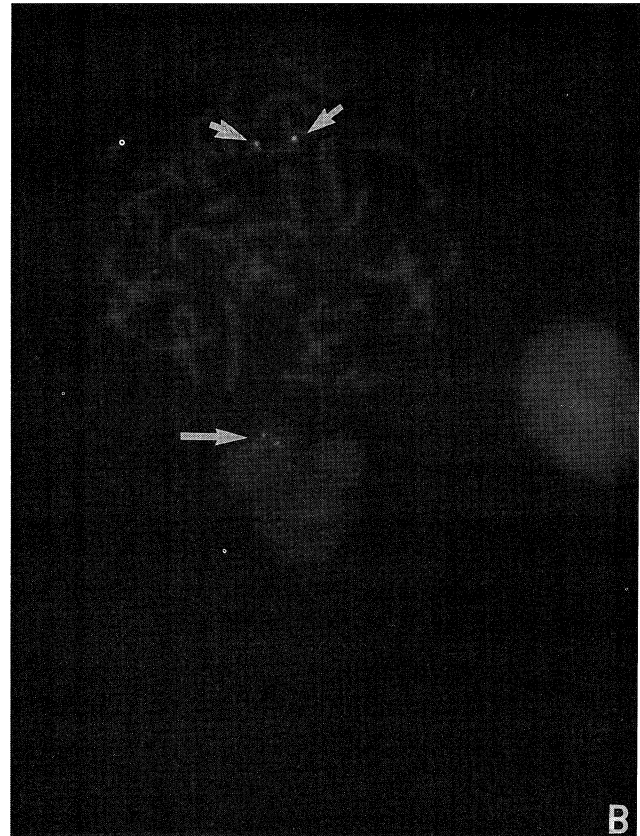
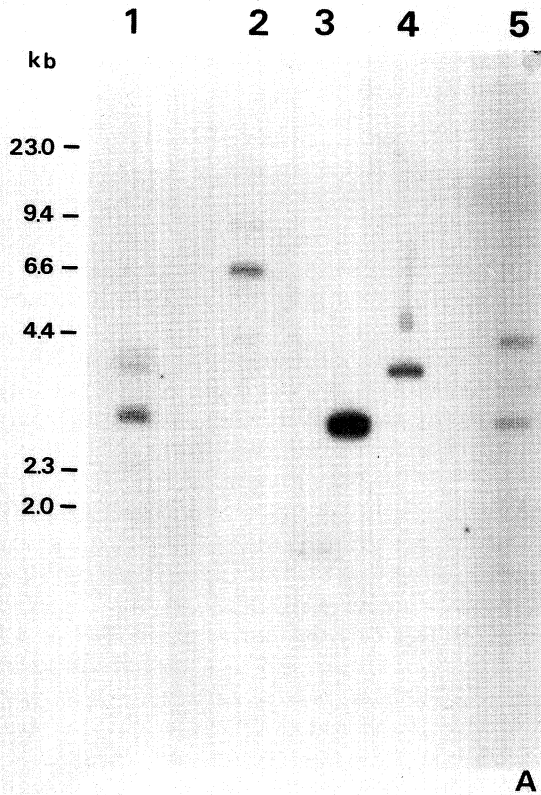


Fig. 3 A: Southern blot of digested human genomic DNA. DNA is digested with SstI (1), SphI (2), HindIII (3), HincII (4) and BamHI (5). The filter has been hybridized to a [32 P] labeled smoothelin cDNA fragment bp 1–600. The probe hybridized only to one or two fragments. B: In situ hybridization with a smoothelin cosmid probe. A signal is displayed in de chromosome spread (short arrows) as well as in the interphase nucleus (long arrow).

report the presence of 'heavy' smoothelin in the vascular tissues of rabbit, pig, dog and men. Wehrens and co-workers showed the presence 'heavy' smoothelin in chicken and demonstrated that the increase in molecular weight is not due to dimer formation or association with another SMC component (Wehrens *et al.* in preparation). The two forms of smoothelin may be a mere reflection of the embryonic origin of the SMC. SMC of the visceral and urogenital tissues are considered to be derived of cells of the mesodermal plate that migrate to form the linings of the tracts of organs such as gut, uterus and prostate (29). In these tissues the 59 kDa isoform of smoothelin is expressed. The origin of the SMC aligning the vascular system is still under debate although the hypothesis that these cells are recruited from the direct surroundings of the vessels under influence of factors produced by the endothelial cells, is now more widely accepted (15, 16). In these cells the 94 kDa isoform of smoothelin is found. Four explanations can be given for the occurrence of two forms of smoothelin: 1) two genes encode the two different forms; 2) alternative

splicing may be responsible for a longer form of the protein in the vascular tissue; 3) different transcriptional regulation (dual promotor) in vascular and visceral tissues; 4) differences in posttranslational modifications may influence the apparent molecular weight of the molecules. The first possibility has been ruled out. In situ hybridization on metaphase spreads with a smoothelin cosmid probe did not show any reproducible signal in addition to the ones on chromosome 22. This is more extensively described in an in situ hybridization study aimed at the assignment of the smoothelin gene to chromosome 22 and its position on the chromosome (Engelen *et al.*, submitted). The FISH results corroborate those of the Southern blotting analysis revealing a banding pattern which is in agreement with smoothelin as a single copy gene in the human genome. The choice between the other three alternatives will need more experimental evidence. However, considering the structure of the putative smoothelin deduced from the known cDNA sequence an extensive posttranslational modification, resulting in an apparent molecular weight

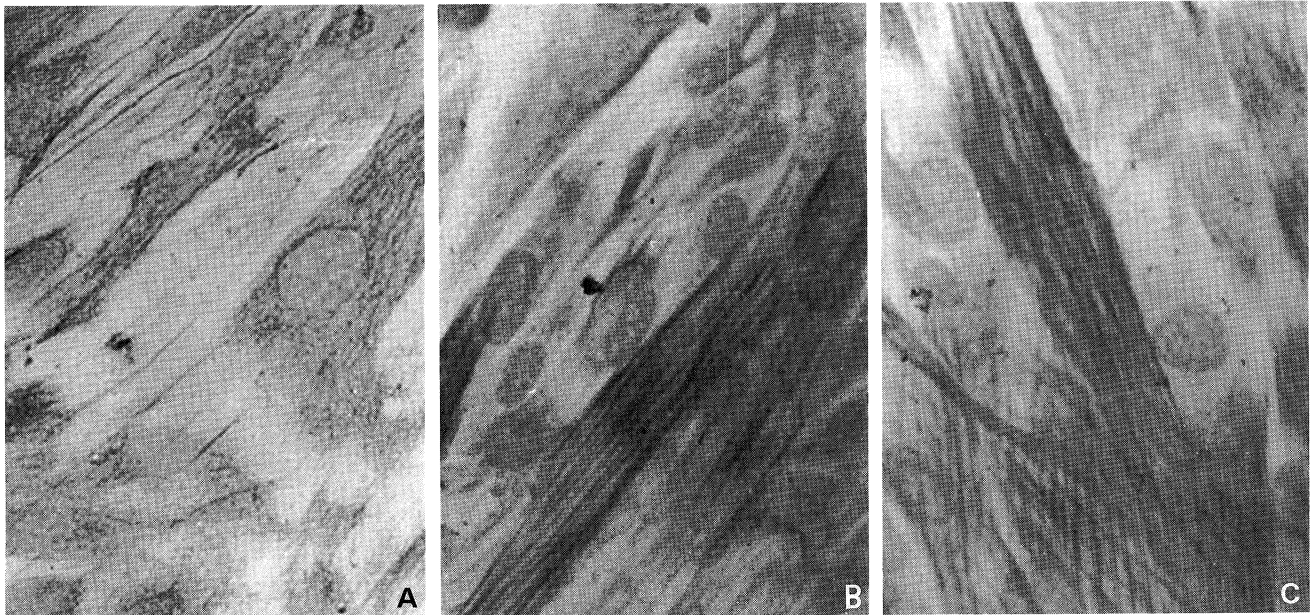


Fig. 4. Second passage of a primary culture of benign prostate hyperplasia cells immunohistochemically stained with rabbit desmin antiserum pDes (A), smoothelin monoclonal antibody R4A (B), and actin monoclonal antibody sm-1 (C). The staining pattern of R4A and sm-1 are similar and differ of the one displayed after incubation with desmin antiserum. Not all cells react.

increase of more than 50%, seems to be unlikely. Therefore, future research will focus on the second and third alternative.

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