Intermediate filaments in smooth muscle from pregnant and non-pregnant human uterus

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The intermediate filament proteins desmin and vimentin from pregnant and non-pregnant uterine muscle and smooth-muscle cells in culture were analysed using SDS/PAGE. The desmin content in uterine muscle increases dramatically during pregnancy, whereas vimentin remains unchanged or changes very little. When muscle cells are kept in culture, a considerable increase in vimentin content is observed as compared with vimentin in freshly isolated non-pregnant uterine tissue. Our results strengthen the view that vimentin and desmin filaments have independent function and turnover, and point to a predominantly structural role for desmin filaments.

INTRODUCTION

Intermediate filaments (IF) are a major filamentous system with a characteristic diameter of 10 nm and are thus intermediate between the actin (6 nm) and myosin (15 nm) filaments (for reviews see [1–5]). A great diversity of IF chains has been found in different cell types, but some degree of similarity between the sequences allows the IF proteins to be classified into four types. Keratins are formed by type I and type II chains; desmin, vimentin and filial fibrillary acidic protein are of type III; and neurofilaments are of type IV. Muscle IF contain type III proteins, namely desmin and vimentin [6–9]. Desmin is the predominant protein of IF in the embryonic and adult forms of skeletal, cardiac and smooth muscles. Vimentin has been described as the major component of IF in mesenchymal cells, but co-exists as a minor component with other classes of IF in several cell types [2,10]. Abundant vimentin filament formation is induced in cells from various origins kept in culture [11,12].

IF have been associated with nuclear surface plasma membrane communication, interaction with cytoplasmic structures, maintenance of cell shape and support of the nucleus and other organelles, and cell differentiation, but their function has not been clearly established. Major changes occur in smooth-muscle cells during pregnancy, and these provide a useful tool for the study of IF.

MATERIALS AND METHODS

Materials

Chemicals for electrophoresis were from Bio-Rad (Watford, Herts., U.K.); proteinase inhibitors and anti-(smooth muscle actin) antibody were from Sigma (Poole, Dorset, U.K.); tissue-culture products were from Gibco (Paisley, Scotland, U.K.); and immunochemicals were from Dakopatts (High Wycombe, Bucks., U.K.). All other chemicals were of analytical grade and were purchased from BDH (Poole, Dorset, U.K.). Disposable plastic material was from Sterilin (Hounslow, Middx., U.K.).

Preparation of protein fractions and extraction of soluble proteins

Fresh myometrial tissue was obtained from patients undergoing either total hysterectomy or elective Caesarian section at full-term pregnancy. Within a few minutes of excision the

tissue was frozen at -70°C . The tissue was cut into small fragments, rinsed with cold 0.9% NaCl and homogenized with three bursts of 15 s each in a Polytron at full power in 20 vol. of buffer (pH 6.8) containing: 60 mM-KCl, 20 mM-imidazole, 0.2 mM-phenylmethanesulphonyl fluoride (PMSF), 1 mg of Tos-Lys-CH₂Cl (*N*-tosyl-L-lysine-chloromethyl ketone; 'TLCK')/l, 1 μg of pepstatin/ml, 10 μg of leupeptin/ml, 10 units of Trasylol/ml, 1 mM-MgSO₄, 1 mM-dithiothreitol and 0.01% NaN₃. The suspension was centrifuged for 15 min at 25000 *g* in a Sorvall RC-5B, the supernatant was removed and the pellet was re-extracted and centrifuged. The supernatants containing soluble cytoplasmic proteins were combined, divided into 100 μl fractions and frozen at -70°C until analysed.

Preparation of cytoskeletal proteins

The pellets from the first preparation were homogenized in buffer containing 20 mM-imidazole, 0.6 M-KCl, 2 mM-EGTA, 1 mM-EDTA, 10 mM-ATP, pH 7.5, and proteinase inhibitors, using one 15 s burst in a Polytron at setting 3. The suspension was centrifuged for 15 min at 25000 *g* in a Sorvall RC-5B, the supernatant was removed and the procedure was repeated. Both supernatants containing the myofibrillar proteins were combined, divided into fractions and frozen at -70°C .

The pellets obtained after the extraction of myofibrillar proteins were extracted twice for several hours in the pH 7.5 buffer modified to contain 1.5 M-KCl and 0.5% Triton X-100 (Surfact-Amps; Pierce). After centrifugation the supernatants were discarded, and the pellets containing cytoskeletal proteins were solubilized in sample buffer, divided into portions and kept at -70°C until needed.

Separation of proteins by electrophoresis

Protein extracts were heated for 5 min at 90°C in the presence of 2% SDS and 2.5% mercaptoethanol, and separated on a 7–20% gradient polyacrylamide gel (30% T, 2.6% C) in the presence of SDS, according to Laemmli [13]. Gels were stained with Coomassie Blue R. Wet gels were scanned in an LKB 2202 UltroScan Laser Densitometer.

Culture of smooth muscle cells

Within minutes of the removal of the uterus, myometrial tissue was dissected from the fundus, minced into small fragments and

Abbreviations used: IF, intermediate filaments; PMSF, phenylmethanesulphonyl fluoride.

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rinsed in phosphate-buffered saline (PBS; KH_2PO_4 , 0.2 g/l; Na_2HPO_4 , 1.15 g/l; KCl, 0.2 g/l; NaCl, 8 g/l) under sterile conditions. The tissue was incubated for 2 h at 37 °C, with no agitation, in PBS containing 50 units of penicillin/ml, 50 µg of streptomycin/ml, 50 mg of gentamycin/ml, 25 mg of fungizone/ml and 0.5 mg of type II collagenase/ml (Sigma). At the end of this incubation time the suspension, containing red blood cells, fibroblasts and some dispersed muscle cells, was discarded and fresh enzyme solution was added to the remaining fragments of tissue. The suspension was vigorously shaken for a few seconds and incubated for 2 h at 37 °C with no agitation, after which the suspension was vigorously shaken again and the remaining clumps were allowed to sediment for a few minutes. Dispersed cells in the supernatant were collected by centrifugation for 10 min at 400 g, washed twice with PBS and resuspended in RPMI 1640 medium containing 30% (v/v) fetal calf serum, 50 units of penicillin/ml, 50 µg of streptomycin/ml, 50 mg of gentamycin/ml and 25 mg of fungizone/ml. Cells were plated at high density in 50 mm Petri dishes and left undisturbed for 3 days, after which time fresh medium and serum were added. Subsequently, the medium was changed every 72 h.

Immunofluorescence

Cells in culture were stained using mouse anti-(smooth-muscle actin) (dilution 1:50) and anti-mouse IgG conjugated to fluorescein isothiocyanate (dilution 1:50) [14]. Cultures were examined with a Leitz phase/fluorescence microscope.

Immunohistochemistry

Histological staining of uterine tissue sections was carried out in the histology and electron microscopy departments at the Clinical Research Centre using DAKO and anti-desmin antibody (dilution 1:60), and standard biotin-avidin peroxidase conjugate and Protein A-gold conjugate methods.

Fig. 1. Analysis of human smooth-muscle insoluble proteins

Myometrial tissue was extracted with low-ionic-strength buffer and the insoluble residue was analysed on SDS/PAGE. Lane a, pregnant uterus; lane b, non-pregnant uterus. V, vimentin; D, desmin; A, actin. M_r standards: 1, 200 000; 2, 116 250; 3, 97 400; 4, 66 200; 5, 42 699.

Fig. 2. Histological staining of myometrial sections

(a) Micrograph of a section of human myometrium stained with anti-desmin antibody and a biotin-avidin-peroxidase conjugate (magnification $\times 87$); (b) negative control using normal mouse serum instead of anti-desmin antibody; (c) electron micrograph of a section of human myometrium labelled with anti-desmin antibody and Protein A-gold conjugate (magnification $\times 8910$); (d) negative control using normal mouse serum instead of anti-desmin antibody.

RESULTS

Fresh myometrial tissue was obtained from patients undergoing either total hysterectomy or elective Caesarian section at full-term pregnancy. Electrophoretic analysis of proteins insoluble in low-ionic-strength buffer showed a marked difference in the desmin content of the pregnant and non-pregnant muscle, whereas vimentin changed very slightly (Fig. 1). The relative proportions of the majority of muscle proteins (cytoskeletal and myofibrillar) in pregnant and non-pregnant uterus remain unchanged (P. Leoni, unpublished work). Vimentin and desmin were identified on Western blots using anti-vimentin and anti-desmin antibodies, followed by a peroxidase conjugate antibody. The pattern of proteins extracted from colon smooth muscle was similar to that from normal uterus (results not shown). In order to verify that no desmin is present in the connective tissue surrounding the muscle cell bundles, histological studies of uterine muscle sections were immunolabelled using anti-desmin antibody and examined by light and electron microscopy. Light microscopy examination of peroxidase-stained sections shows the presence of desmin (dark stain) only inside muscle cells (Fig. 2a). Electron microscopy using Protein A-gold conjugate also showed that no desmin (black dots) is located outside the cells (Fig. 2c).

After exhaustive extraction of uterine tissue with buffers of increasing salt concentration, the cytoskeletal protein residue

Fig. 3. Analysis of cytoskeletal proteins from myometrial tissue

Cytoskeletal proteins were separated on SDS/PAGE, the gels were stained and the bands intensity was measured by densitometry. Only the relevant part of the gel is shown. Lane a, pregnant uterus; lane b, normal uterus. An immunofluorescent stain of smooth muscle actin filaments is shown. A, actin; D, desmin.

Fig. 4. Immunofluorescent stain of smooth-muscle actin filaments

Myometrial smooth muscle cells in primary culture were labelled using the monoclonal antibody anti-(smooth muscle actin) and a fluorescein conjugate, and examined with a fluorescence/phase microscope. (a) Fluorescence micrograph; (b) phase-contrast micrograph (magnification $\times 254$ in both cases).

Fig. 5. Insoluble proteins from smooth-muscle cells in culture

Smooth muscle cells from primary cultures were extracted with low-ionic-strength buffer. The insoluble residue was analysed on SDS/PAGE, together with a similar residue from normal myometrial tissue. Lane a, smooth cells in culture; lane b, normal uterus. V, vimentin; D, desmin; A, actin. M_r standards: 1, 97 400; 2, 66 200; 3, 42 699; 4, 31 000; 5, 21 500; 6, 14 400.

was analysed on polyacrylamide gels (Fig. 3). Although a high proportion of actin is removed during the extraction with buffer containing 0.6 M-KCl, some of it remains associated with the cytoskeletal protein even after repeated extractions with buffer containing 1.5 M-KCl. In five preparations, the ratio of desmin to actin in normal uterus is 0.56 ± 0.07 whereas in pregnant uterus it is 2.64 ± 0.26 , as determined by densitometry. The amount of actin, in proportion to other myofibrillar proteins, does not seem to change in pregnant compared with non-pregnant tissue.

Primary cultures of smooth-muscle cells were established and kept in culture for 3 weeks in the presence of 10 nM-oestradiol, 10 nM-progesterone and 2% bovine embryonic extract. The homogeneity of the culture was tested by immunofluorescence (Fig. 4) using anti-(smooth-muscle actin) antibody. The proteins from the cells in culture which were insoluble in low-ionic-strength buffer were analysed on polyacrylamide gels and compared with the same proteins extracted from normal uterine tissue. As demonstrated for other cell types in culture, smooth-muscle cells show a considerable increase in vimentin content, whereas there is very little change in desmin content (Fig. 5). Cells in these cultures divide actively, but show hardly any change in size.

DISCUSSION

During the process of differentiation of uterine stromal cells after the onset of pregnancy (decidualization), desmin expression increases markedly. This has been proposed to be due to some myogenic characteristic occurring during decidualization or to the possibility that desmin expression may be a characteristic of

differentiation in a variety of cells [15]. However, findings suggesting that the formation of a cytokeratin network in mouse embryos is unnecessary for the initial stages of differentiation [16] weaken the interpretation that IF have a role in cellular differentiation.

The reorganization of desmin from a longitudinal to a transverse network around the Z-lines during myogenesis has led some authors to propose that IF may have an important role in bringing the Z-disks into lateral register [17]. Other studies, using mutants which do not possess organized microfibrils, showed that the organization of myofibrils in register is independent of the transverse desmin distribution [17]. Recently it has been proposed that titin filaments act as integrators during skeletal muscle development [18].

Desmin content in the uterine muscle increases considerably in pregnant uterus at term. The proportion of actin that seems to remain associated with cytoskeletal proteins after extraction of myofibrillar proteins is considerably lower in pregnant uterine muscle than in normal uterus. It would seem that, although actin can form complexes with desmin [19], a higher amount of desmin remains in pregnant uterus which is not associated with actin. Desmin content does not change during culture of smooth-muscle cells, even though cells are metabolically very active, as indicated by their protein and DNA synthesis rates measured with radioactive precursors.

The increase in desmin content correlates with the dramatic increase in the cell size that occurs during pregnancy. An increase in size also occurs in stromal cells during decidualization. This would suggest that desmin filaments have a purely structural role, maintaining the cell shape and providing mechanical support to cell organelles. Vimentin is tightly associated with the cell nucleus. It has the ability to bind to lamin B on the nucleus surface, and might assemble vectorially towards the plasma membrane [20,21]. Vimentin content changes very little after the uterus becomes hypertrophic, but changes considerably after the cells are kept in culture, indicating a more specific involvement in cell motility and attachment to a substrate. Intermediate filament function still requires much investigation, and use of an ap-

propriate animal model, that allows sequential studies after the onset of pregnancy, should prove very informative.

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