Expression and organization of sarcomeric constituents during muscle cell differentiation

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EXPRESSION AND ORGANIZATION OF
SARCOMERIC CONSTITUENTS DURING
MUSCLE CELL DIFFERENTIATION

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EXPRESSION AND ORGANIZATION OF SARCOMERIC CONSTITUENTS DURING MUSCLE CELL DIFFERENTIATION

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Rijksuniversiteit Limburg
te Maastricht, op gezag van de Rector Magnificus, Prof. Dr. H. Philipsen,
volgens het besluit van het College van Dekanen, in het openbaar
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The investigations described in this thesis were carried out at the Department of Molecular Cell Biology & Genetics, Cardiovascular Research Institute Maastricht (CARIM), University of Limburg, Maastricht, and partly at the Department of Pathology, University Hospital Nijmegen.

CONTENTS

List of abbreviations	6
General Introduction	7
Chapter 1: Desmin and titin expression in early postimplantation embryos	15
Chapter 2: Baby hamster kidney (BHK21/C13) cells can express striated muscle type proteins	31
Chapter 3: Differentiation of human skeletal muscle cells in culture: maturation as indicated by titin and desmin striation	49
Chapter 4: Characterization of cardiotin, a structural component in the myocard	65
Chapter 5: Tissue-specific expression of a vimentin-desmin hybrid gene in transgenic mice	85
Chapter 6: Transgenic expression of the muscle-specific intermediate filament protein desmin in nonmuscle cells	99
Chapter 7: Disruption of intermediate filaments by expression of dominant negative desmin mutants in transgenic mice	121
Summary/Samenvatting	141
Dankwoord	147
Curriculum vitae	149
Publications	151

ABBREVIATIONS

BHK	: baby hamster kidney
bp	: basepairs
Da	: Dalton
DMEM	: Dulbecco's modified Eagle's medium
d.p.c.	: days post coitum
EDTA	: ethylenediaminetetra acetic acid
EMEM	: Eagle's modified minimum essential medium
FCS	: fetal calf serum
FITC	: fluorescein isothiocyanate
GFAP	: glial fibrillary acidic protein
HBE	: human brain extract
IF(s)	: intermediate filament(s)
IFP(s)	: intermediate filament protein(s)
kbp	: kilobasepairs
kDa	: kiloDalton
NaDOC	: sodium deoxycholate
PAA	: polyacrylamide
PBS	: phosphate buffered saline
PMSF	: phenylmethylsulfonyl fluoride
SDS	: sodiumdodecyl sulphate
SR	: sarcoplasmic reticulum
TR	: Texas red
TRITC	: tetramethylrhodamine isothiocyanate

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Muscle cells are of mesodermal origin. Three types of muscle tissue may be distinguished in mammals on basis of morphologic and functional characteristics [28]:

1. Smooth muscle tissue consists of collections of fusiform cells, which do not show intracellular striation. Smooth muscle cells normally exhibit relatively slow uncontrolled contractions, and are for example found in the large and small intestine, the esophagus, the stomach and around blood vessels.
2. Skeletal muscle is composed of bundles of very long, cylindric, and multinucleated cells that show cross-striations. Their contraction is quick, forceful, and usually under voluntary control. Skeletal muscle cells comprise the somatic musculature of body wall and extremities.
3. Cardiac muscle is composed of elongated or branched individual cells that often run parallel to each other and show cross-striations. Individual cardiomyocytes are linked to each other at the intercalated disks, structures only found in cardiac muscle. Cardiac muscle is involuntary, vigorous, and rythmic.

The sarcoplasm of both types of striated muscle cells contains long, cylindrical filamentous bundles called myofibrils, which have a diameter of 1-2 μm and run in the long axis of the muscle cell. Myofibrils consist of sarcomeres, the contractile units of striated muscle cells.

Figure 1: Schematic drawing of a striated muscle sarcomere

Sarcomeric structure

When examined by light microscopy, sarcomeres show regular dark and light bands, called A-bands and I-bands, respectively. A narrow line, the Z-disc borders at both sides of a sarcomere. With the electron microscope two types of filaments become evident in

sarcomeres, i.e. thick filaments (15 nm) known to consist of myosin, and thin filaments (8 nm) containing actin, tropomyosin, and troponin. Thick and thin filaments overlap for some distance within the A-band. In the center of the A-band a lighter zone can be seen, called the H-band, which consists of only thick filaments, this in contrast to the I-band that is mainly composed of thin filaments. The M-line lies in the centre of the H-band.

Molecular composition of the sarcomere

In the sarcomere the functional proteins mentioned above are strictly organized [10, 15]. The two major proteins of the sarcomere, myosin with an approximate molecular weight (MW) of 200 kDa and actin (MW: 42 kDa), together represent 55% of the total protein content of striated muscle [28], and drive contraction [15].

The 95 kDa protein α -actinin [8, 12], a major component of the Z-disc, is thought to anchor the actin filaments to this region. The intermediate filament protein desmin (MW: 53 kDa), located in the outer circumference of the Z-disc, keeps the sarcomeres in register by linking the myofibrils to each other or to the sarcolemma [23, 50, 51]. In cardiac muscle desmin is not only observed in the Z-disc, but also in the intercalated disc, in close association with the desmosomes [16]. Like desmin also α -actinin is present in the intercalated disc.

Two muscle-specific, giant sarcomeric proteins i.e., titin (MW: approximately 3.000 kDa) [36, 37] and nebulin (MW: approximately 800 kDa) [60], play probably an important role in regulating assembly of thick and thin filaments, respectively [18, 34, 56, 63]. Titin molecules, found in both skeletal and cardiac muscle are over 1 μ m long [18], and span the distance from M-line to Z-disc [19]. The titin protein comprises about 10% of the myofibrillar mass [55]. Titin filaments may play an important role in elasticity and enzymatic activity of the myofibrils [56], as well as in maintaining the alignment of myosin filaments [26]. Nebulin is found in skeletal muscle and not in cardiac muscle, and acts as a protein-ruler, precisely regulating thin filament assembly [27, 33, 34].

Other sarcomeric proteins are the actin associated proteins [15] such as tropomyosin (MW: 35 kDa) [6, 29], troponin I and troponin T (MW: 22 kDa, 37 kDa, respectively) [9], and vinculin (MW: 130 kDa) [39].

The contractile proteins of striated muscle described above are often represented by

Figure 2: Electron microscopic photograph of human skeletal muscle sarcomeres. Bar indicates 600 nm. Z: Z-disc; A: A-band; I: I-band; H: H-band; M: M-line. (Photograph kindly provided by Dr. Paul Jap, Department of Cell Biology & Histology, University of Nijmegen, The Netherlands)

families of very similar isoforms, which are functionally unique in developing and mature muscle cells [2].

Differentiation related protein expression and assembly *in vivo* and *in vitro*

During *in vivo* and *in vitro* myogenesis of smooth, skeletal, and cardiac muscle cells, different sets of muscle specific proteins and intermediate filament proteins occur precisely at different stages of differentiation, in an ordered sequence [1, 2, 10, 11, 15, 19, 24, 25, 46, 47, 52, 53, 56, 57, 58, 59, 61, 62].

Intermediate filament proteins are expressed in early stages of myogenesis [15, 46, 54, 57] where keratins are the first expressed in embryogenesis. During further differentiation keratin expression decreases and is superseded by expression of another intermediate filament protein, usually vimentin [54]. Vimentin is found in presumptive replicating myoblasts or stages of development when desmin is still undetectable [21]. Desmin is found in all muscle cell types and can coexist with vimentin in early stages of myogenesis [20, 46, 57, 58]. In general, however the concentration of desmin increases during the differentiation process of myocytes *in vivo* and *in vitro*, when the vimentin filaments gradually disappear [3, 15, 40, 46, 52, 57, 58]. After myofibril assembly in skeletal and cardiac muscle, desmin is distributed in a cross-striated pattern, situated in the Z-line and the intercalated disc [4, 38, 46, 47, 57, 58].

Titin is expressed in a characteristic punctate fashion even before desmin appears in differentiating myoblasts. This phenomenon is described for elongating skeletal muscle and myocard cells *in vivo* and *in vitro* [7, 20, 25, 46, 53, 57]. During the differentiation process, when myoblasts form myotubes, the titin spots rearrange from a random distribution to a localization associated with the myofibrils, which contain sarcomeric α -actin and α -actinin, but not desmin or myosin [58, 59]. Further in the differentiation process assembly of titin molecules into the sarcomeres of skeletal and cardiac muscle cells takes place, indicated by the typical cross-striated pattern seen with antibodies to titin [18, 20, 46, 58]. After the appearance of titin cross-striations other muscle specific proteins, such as desmin, tropomyosin and myosin assemble into the sarcomere in an ordered fashion [46, 58, 59]. Apparently titin serves as a template for assembly of other sarcomeric proteins during myofibrillogenesis. At later stages of development nebulin is integrated in the already formed sarcomeric structures of skeletal muscle cells [20, 59].

Transgenic mouse models for the study of muscle protein function

Transgenic mouse models have been used to study the function of muscle proteins [14] and that of intermediate filament proteins in particular. Modified hamster desmin genes, driven by hamster vimentin and hamster desmin promoters, were introduced into the germ line of mice [32, 41]. Since intermediate filament proteins are expressed in a developmentally regulated and tissue-specific fashion, alterations in highly conserved protein domains, that may be of crucial importance for proper functioning of intermediate filaments, are presumed not to be tolerated in normally functioning cells. The amino acid sequence of the carboxy-terminal domain of vimentin and desmin are much more conserved among species than the sequence of the amino-terminal domain and approximates the conservation of the rod domain [42, 64]. The amino-terminal part of the different intermediate filament subunits seems to be of major importance in regulating filament assembly [5, 13, 17, 30, 49, 54] and vimentin or desmin gene constructs containing modifications or deletions in the carboxy-terminal part of the α -helical 'rod' domain, disrupt intermediate filament networks in cultured cells in a dominant-negative fashion. This disruption of the intracellular intermediate filament networks can occur without affecting cell morphology, motility, or cell division [22, 31, 35, 43, 44, 45, 48].

Until now, no developmental or morphological abnormalities could be detected in transgenic mice using truncated desmin and vimentin genes.

Aim of this study

To understand the process of assembly of muscle-specific proteins during myofibrillogenesis *in vivo* and *in vitro* and the interaction between these proteins in different stages of muscle development. Immuno-localization studies were performed in differentiating muscle cell cultures, as well as in mouse embryos and normal adult tissues of different species.

Moreover, to study the correlation between the function and molecular organization of these molecules, transgenic mice with a disorganized intermediate filament pattern were extensively studied at different molecular and morphological levels.

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CHAPTER 1

DESMIN AND TITIN EXPRESSION IN EARLY POSTIMPLANTATION MOUSE EMBRYOS

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and F.C.S. RAMAEKERS**

Development 107, 585-596, 1989

ABSTRACT

The expression of the intermediate filament (IF) constituents desmin, vimentin and keratin, as well as the striated-muscle-specific marker titin, was studied in mouse embryos of 8.0 to 9.5 days post coitum (d.p.c.), using the indirect immunofluorescence technique in combination with polyclonal and monoclonal antibodies. During the development of the embryo, desmin was first detected at 8.25 d.p.c. in the ectoderm, where it was transiently coexpressed with keratin and vimentin. At later stages, the ectoderm contained only keratin and to a certain extent also vimentin IF. At 8.5 d.p.c., desmin was found exclusively in the heart rudiment, and remained present with increasing intensity in the myocardial cells during later cardiogenesis. Striation of desmin in the heart muscle cells was observed in 9.5 d.p.c. embryos. At these stages (8.5 - 9.5 d.p.c.), triple expression of the IF proteins desmin, vimentin and keratin was evident in these cells. From 9.0 d.p.c. onwards, desmin could be detected in the myotomes as well. Immunoblotting studies of 9.5 d.p.c. mouse embryos confirmed the immunohistochemical data.

Titin was found in the early heart anlage at stage 8.25 d.p.c., when no desmin expression was observed in this tissue. At this stage the titin appeared in a punctate pattern, similar to that observed in cardiac myofibrils of early chicken embryos (Tokuyasu and Maher: *J. Cell Biol.* 105, 2781-2793, 1987). In 8.5 d.p.c. mouse embryos, this punctate titin staining pattern was still observed, while, at this stage, a filamentous staining reaction could be seen with the desmin antibodies. During further development, cross-striation was detected within myocardial cells using the polyclonal titin antibody from 9.0 d.p.c. onwards, i.e., before such striation could be detected with the desmin antibodies.

From these data, we conclude that titin synthesis may anticipate desmin expression in the developing mouse myocard, although the level of expression of the former protein remains low until 9.0 d.p.c.

INTRODUCTION

The protein constituents of intermediate-sized filaments of the cytoskeleton in cells of adult animals have been described to be expressed in a more or less tissue-specific fashion [33]. Keratin normally occurs only in epithelial tissues, while neurofilaments and glial fibrillary acidic protein (GFAP) are mainly restricted to neural cell types [5, 39; for review see: 15]. Desmin has so far been found to be virtually muscle specific [6; for exceptions see: 10, 27], while vimentin occurs in cells of mesenchymal origin, but is also occasionally coexpressed with the other intermediate filament proteins (IFP) [9]. The expression of IFP in developing embryos has been studied by several investigators [see review: 46]. From these results, it has become obvious that, apart from the lamins, cytokeratins are the first cytoplasmic IFP to be expressed in early embryogenesis [18]. Embryonal cells containing this type of IFP are multipotential in nature and switch on the expression of vimentin when differentiating into mesenchymal cells [9, 46]. Subsequently, differentiation into mature cells results in the expression of the more specific IFP, such as desmin in muscle cells and GFAP in glial cells. However, coexpression of vimentin with these IFP is seen during embryogenesis and even after birth [24, 31, 38]. During myogenesis vimentin and desmin are expressed in sequence in differentiating myocytes [8]. In replicating myoblasts, vimentin is found in early stages of development at which desmin is undetectable. When the myogenic cells withdraw from the cell cycle at an early stage of myofibrillogenesis, desmin expression is initiated [8]. As muscle cell differentiation proceeds, desmin expression becomes more prominent, while its localization becomes more and more restricted to the outer circumference of the Z-band, both in skeletal and cardiac striated muscle cells [4, 8, 14]. Most of the work on

IFP expression in muscle morphogenesis has concentrated on avian tissues [1, 41, 44], while mammalian myofibrillogenesis has hardly been studied at this level. Since fundamental differences have, however, been noted during myogenesis in avians and mammals [2, 21], we have studied desmin and vimentin expression in mouse embryos at 8.0 to 9.5 days post coitum (d.p.c.). It was postulated that during avian *in vivo* and *in vitro* myofibrillogenesis desmin is one of the earliest known markers for cells in the myogenic lineage and that proteins such as titin and myosin heavy chain occur later. We also studied the development of titin, which has been identified as a specific component in cardiac and skeletal muscle [25, 26, 50].

MATERIALS AND METHODS

Embryos

The embryos used for this study were from spontaneous matings of mice of the C75bl/CBA (BCBA) strain. The presence of a vaginal plug indicated a successful mating. The conceptuses were thus presumed to be 0.5 days old at noon (12:00) on the day the vaginal plug was found. At 8.0, 8.25, 8.5, 9.0 and 9.5 days post coitum (d.p.c.) embryos were dissected from the uterus and washed in cold phosphate buffered saline (4°C; PBS: 137 mM sodium chloride (Merck, Darmstadt, FRG); 13 mM di-sodium hydrogen phosphate dihydrate, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (Merck); 3mM potassium dihydrogen phosphate, KH_2PO_4 (Merck), pH 7.4). Embryos were quickly frozen in liquid N_2 after removal from the uterus and immersed in Tissue Tek (Miles Inc., Elkhart, IN, USA). The developmental stages are designated according to Theiler [42].

Indirect immunofluorescence microscopy

Five micrometer thick cryostat sections were air-dried, fixed with methanol (Merck) at -20°C for 5 min, followed by acetone (Merck) fixation at -20°C for 1 min. After air-drying for 15 min at room temperature, the sections were incubated with the primary antibody for 30 min at room temperature, and washed three times (5 min each) in PBS. Then they were incubated with the secondary antibody for 30 min at room temperature and again washed three times (5 min each) in PBS, for 5 min in distilled water and finally for 5 min in methanol. Sections were mounted in Mowiol (Hoechst, Frankfurt, FRG) containing 2.5% (w/v) NaN_3 (Merck) to retard fading [19]. Slides were examined using a Zeiss Universal microscope equipped with epi-illumination optics.

Gel electrophoresis and immunoblotting

Cytoskeleton preparations from 9.5 d.p.c. mouse embryos were made as follows. After dissection of the embryos from the uterus, a high-salt buffer (1.5 M KCl (Merck); 0.5% Triton X-100 (BDH Chemicals Ltd., Poole, UK); 5 mM EDTA (Merck), 0.4 mM phenylmethylsulfonyl fluoride (PMSF; Merck) and 10 mM Tris-HCl (Merck), pH 7.2) extraction was performed for 5 min at 4°C by homogenization of the embryo in a Dounce potter. The pellet obtained by centrifugation for 5 min at 12,000xg was washed with cold (4°C) low-salt buffer (5 mM EDTA, 0.4 mM PMSF and 10 mM Tris-HCl, pH 7.2), essentially as described [28]. After a second washing step in PBS, the cytoskeleton preparation was dissolved by boiling for 5 min in SDS-sample buffer [23].

One-dimensional gel electrophoresis was performed in 10% polyacrylamide slab gels containing 0.1% SDS (BDH Chemicals Ltd. [23]). To compare the amounts of protein loaded on each lane, gels were stained with Coomassie Brilliant Blue R250 (Gurr, Hopkin

and Williams, Chadwell Heath, Essex, UK) as described [3]. Two-dimensional gel electrophoresis was performed essentially as described by O'Farrell [30]. In the first dimension, isoelectric focusing was performed in 4% polyacrylamide (Bio-Rad Laboratories, Richmond, CA, USA) rod gels containing 1% Biolyte, pH 3.5 - 10 (Bio-Rad Laboratories). For the second dimension, the rod gels were applied directly onto the stacking gel of SDS-polyacrylamide gels.

For immunoblotting experiments, the electrophoretically separated polypeptides were transferred to a nitrocellulose sheet (Schleicher & Schuell Membrane Filters BA 85, Dassel, FRG) by blotting for 1 h at 100 V in a cold (4°C) buffer containing 25 mM TRIS-HCl, 192 mM glycine (Merck), 0.02% SDS and 20 % methanol (pH 8.3) [54]. The blots were incubated for 90 min with PBS containing 0.05% Tween20 (Sigma Chemical Company, St Louis, USA). All reagents were diluted in this solution, which was also used for the washing steps. After incubation overnight with undiluted culture supernatants of the primary antibodies RD301, RV202, CK18-2 or BV1118, the blots were washed three times for 10 min and incubated for 1 h with peroxidase conjugated rabbit anti-mouse Ig (DAKOpatts, Glostrup, Denmark) diluted 1:400. The blots were washed again three times for 10 min in 0.05% Tween20/PBS and once in PBS alone for 10 min and stained with 4-chloro-1-naphthol (Merck) and 0.12 % hydrogen peroxide (Merck). After staining the blots were rinsed for 5 min with water.

Antibodies

The following antibody preparations were used in this study:

1. A polyclonal antiserum to chicken gizzard desmin (pDes). Preparation and characterization of this rabbit antiserum have been described elsewhere [35]. For indirect immunofluorescence microscopy, this serum was diluted 1:50 in PBS.
2. Two mouse monoclonal antibodies to desmin (DE-B-5 and DE-R-11; DAKOpatts) [6]. These antibodies were used as undiluted culture supernatants in the immunohistochemical assays.
3. A mouse monoclonal antiserum to desmin (RD301), giving a weak, although specific, reaction on muscle cells in adult and embryonic mouse when used in the indirect immunofluorescence assay. This antibody has been described before [32].
4. An affinity-purified polyclonal antiserum to bovine lens vimentin (pVim). Purification and characterization of this polyclonal rabbit antiserum have been described in detail elsewhere [36].
5. A mouse monoclonal antibody (BV1118) of the IgM subclass, reactive with vimentin of human, bovine, rabbit, hamster, mouse and chicken origin (C. Viebahn, unpublished data). This antibody, when tested in the indirect immunofluorescence assay, stains tissues of mesenchymal origin such as fibroblasts, endothelial cells, some smooth muscle cells, glomeruli in the kidney, but does not stain most adult epithelial cells. In cultured cells (BHK-21/C13, HeLa), a filamentous staining pattern is obtained with antibody BV1118. No significant reaction is found in cultured cells such as T24, RT4 and MCF-7, known to be vimentin negative [13]. In one- and two-dimensional immunoblotting assays of cytoskeletal extracts from bovine lens, BHK-21/C13 cells (Fig. 1), and HeLa cells, this monoclonal antibody shows a positive reaction with a protein band migrating at the molecular weight level and isoelectric pH of vimentin. This antibody was used as undiluted culture supernatant in the immunohistochemical assays.
6. A mouse monoclonal antiserum to vimentin (RV202), giving a weak, although specific, reaction on stromal cells and other mesenchymal tissues in adult and embryonic mouse in the indirect immunofluorescence assay, was used for immunoblotting experiments. This antibody has been described in detail elsewhere [32, 34, 48].

Figure 1: Characterization of monoclonal antiserum BV1118 by 1- (A, C, E) and 2-dimensional (B, D, F) immunoblotting on cytoskeletal preparations of bovine lens (A, B) and BHK-21/C13 cells (C-F). Note that antiserum BV1118 reacts exclusively with vimentin (v) and its breakdown products (A-D), both from calf and hamster. Reincubation of the immunoblots with the monoclonal desmin antiserum (RD301; E, F) confirms the vimentin-specific reaction of antiserum BV1118. d: desmin

7. An affinity-purified polyclonal antiserum to human skin keratins (pKer). This rabbit antiserum is described elsewhere [36]. For indirect immunofluorescence microscopy, this antiserum was diluted 1:10 in PBS.
8. A mouse monoclonal antibody (CK18-2) specifically recognizing cytokeratin 18 was raised against human mesothelial cells. This antibody was used as undiluted culture supernatant [3].
9. An affinity-purified polyclonal rabbit antiserum to titin, isolated from *Physarum polycephalum* (kind gift from Dr. D. Gassner, Bonn, FRG), was used in a 1:25 dilution. Preparation and specificity of this antiserum have been described by Gassner [11]. When tested on adult mouse tissues using the indirect immunofluorescence assay, this antiserum reacted specifically with striated (skeletal- and cardiac-) muscle cells. No reaction was found in smooth muscle cells or in nonmuscle cells.
10. A polyclonal antiserum against human fibronectin was purchased from DAKOpatts and used at a dilution of 1:120.

As secondary antibodies fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, goat anti-mouse IgM and goat anti-rabbit IgG were used in a dilution of 1:60. All FITC-conjugated antisera were obtained from Nordic Immunochemicals (Tilburg, The Netherlands).

In order to prove that the correct protein constituents are recognized in mouse embryos, studies were performed with 9.5 d.p.c. embryos. Fig. 2 shows that with the monoclonal antisera to vimentin, desmin and keratin 18, these individual components can be detected in cytoskeletal preparations from 9.5 d.p.c. embryos.

RESULTS

The results obtained in the immunohistochemical studies, performed on mouse embryos of 8.0, 8.25, 8.5, 9.0, and 9.5 d.p.c., are summarized in Table 1 and depicted in figures 3-9.

Presomite embryo (Theiler stage 11)

At 8.0 d.p.c., the polyclonal antiserum pKer shows a strongly positive reaction in the visceral endoderm. An extremely intense reaction with this antiserum was also observed

Figure 2: Immunoblotting study on cytoskeletal preparations from 9.5 d.p.c. mouse embryos (lanes A-D). Immuno-blots were incubated as follows: lane A) with the monoclonal desmin antiserum (RD301); lane B) with the monoclonal vimentin antiserum (RV202); lane C) with RD301 and subsequently with RV202; lane D) as lane C, but thereafter incubated with the monoclonal keratin 18 antiserum (CK18-2). For comparison, lane E) depicts an immunoblot of a chicken gizzard desmin preparation incubated with RD301 and lane F) a cytoskeletal preparation of cell line RT4 incubated with CK18-2, to show the position of desmin (d) and keratin 18 (k), respectively. v: vimentin.

embryo (Fig. 3) in which the heart-forming region can be seen to have bent ventrally in order to form the foregut pocket. In the sagittal sections through this region, we have observed no reaction, or at best an extremely weak staining reaction, with the desmin polyclonal antiserum (pDes; Fig. 3A). However, the basal plate of the neuroectoderm overlying the foregut pocket dorsally showed a positive staining reaction with the desmin antiserum while the mesoderm and endoderm were negative. Using a polyclonal titin antiserum (pTitin), single positive dots were observed in the heart rudiment (Fig. 3B). The mesoderm, the ectoderm, the endoderm and the foregut pocket were negative. The vimentin monoclonal antiserum (BV1118; Fig. 3C) and the polyclonal antiserum against vimentin (pVim) were positive in the mesoderm and the neuroectoderm as well as in the heart anlage. In particular with the monoclonal vimentin antiserum (Fig. 3C), a strong filamentous staining was found in the heart anlage. Note that there is also a strong positive staining reaction in the neuroectoderm overlying the foregut pocket, indicating coexpression with desmin in these cells. An intense reaction with the monoclonal antibody against cytokeratin 18 (CK18-2; Fig. 3D) was observed in the visceral endoderm, the foregut pocket and the visceral layer of the yolk sac at this stage. Except for the amnion epithelium, both ectoderm and neuroectoderm are still negative for the monoclonal and polyclonal keratin antibodies. However, mesodermal cells anterior to, and in, the heart anlage show a distinct positive reaction for keratin (Fig. 3D). Fibronectin showed the outlines of the basement membrane of the endoderm and the amnion epithelium, and was detected in the mesenchyme (Fig. 3E), as well as in the heart anlage. The endoderm and ectoderm are negative with this antiserum.

A slightly more advanced stage of heart development is shown in Fig. 4 in horizontal sections of a 8.5 d.p.c. mouse embryo. Cells of the presumptive endocard can now be seen between the endoderm and the developing myocard. Desmin is now exclusively observed in the developing heart (Fig. 4A), and could not be detected in the

in the parietal endoderm, while the mesoderm and ectoderm were virtually negative. Antibody CK18-2 reacted in a similar fashion but weaker. The vimentin antiserum (pVim) showed a pronounced reaction in the mesoderm, which was particularly obvious in the tangentially sectioned areas of the embryos. The polyclonal desmin antiserum (pDes) was negative in all embryonic tissues, but did stain the extraembryonic smooth muscle tissue of the uterus. Fibronectin was detected extracellularly in the embryonic mesoderm and in nonembryonic tissues, but not in the visceral endoderm and ectoderm. The polyclonal titin antiserum (pTitin) was also tested at 8.0 d.p.c. and found to be negative.

Heart rudiment (Theiler stage 12)

In the mouse, heart development starts in the midline as an unpaired anlage [21] rostral from the neural plate and caudal from the embryonic-extraembryonic junction [42]. This early stage of heart formation was studied in a 8.25 d.p.c.

Table 1: Early expression of desmin and titin in the developing heart of mouse embryos.

Stages of cardiogenesis (Theiler stage)	Heart rudiment 8.25 d.p.c. (12)	Heart rudiment 8.5 d.p.c. (12)	Tubular heart 8.5 d.p.c. (13)	Segmented heart 9.0 d.p.c. (14)	Segmented heart 9.5 d.p.c. (14)
desmin expression pattern	-	+ filaments	+ filaments	+ filaments	+ filaments and striation
titin expression pattern	+ dots	+ dots	+ filaments	+ filaments and striation	+ filaments and striation

neuroectoderm or any other region of the embryo. Titin could be detected in the developing heart region, again in a punctate distribution (Fig. 4B). Vimentin was coexpressed with desmin in this region, and showed a strong filamentous immunofluorescence staining reaction in the myocardial cells (Fig. 4C), but virtually no reaction in the endoderm. Underlying the endoderm, a few vimentin positive, but desmin negative, cells can be identified as the first endocardial cells. The neuroectoderm was still positive for vimentin (results not shown). Both the polyclonal and monoclonal keratin antisera stained cells of the heart anlage (Fig. 4D) as well as the overlying endoderm, while the neuroectoderm was negative.

Tubular heart (Theiler stage 13)

The next stage during heart development is the formation of a tubular heart in which the endocard is almost completely surrounded by myocard. Fig. 5 shows oblique sections through such a 8.5 d.p.c. embryonic heart. The formerly punctate staining pattern of titin antibodies is now replaced by a more filamentous staining pattern (Fig. 5B). The desmin-staining reaction remains filamentous, comparable to the foregoing developmental stage (Fig. 5A). With the monoclonal and polyclonal vimentin antisera (Fig. 5C), a strong positive reaction was observed in the myocard and the endocard. Also with the keratin antisera we have found a positive reaction in the myocard of the tubular heart (result not shown).

Segmented heart (Theiler stage 14)

At this stage, the anlagen of the different parts of the heart can be distinguished, i.e. the anlagen of the ventricular and the atrial compartments (Fig. 6). As in 8.5-day-old embryos, in 9.0 d.p.c. mouse embryos desmin expression seems to be restricted to the heart tissue, except for the myotomes (see below). The cells forming the myocardial wall are strongly positive with the polyclonal desmin antiserum (Fig. 6A), while the endocardial tissues are distinctively negative (asterisks in Fig. 6A). The monoclonal desmin antibody DE-B-5 showed only a partial reaction in the myocardial wall (result not shown). An extensive staining reaction was found in all cells of the myocardial wall with the titin antiserum (Fig. 6B), which was negative in the endocardial cells. In some of the myocardial cells, a transverse cross-striation was found (Fig. 7B) with this polyclonal titin antiserum, while there was no cross-striation found with the desmin (Fig. 7A) and the vimentin antibodies at this stage. The endocardial cells as well as the myocardial cells are specifically stained with the vimentin antiserum (Fig. 6C). Keratin expression seems to be

Figure 3: Immunofluorescence micrographs of sagittal frozen sections from 8.25 d.p.c. embryos of the mouse (Theiler stage 12) showing the heart rudiment (HR), the foregut pocket (FP) and the neuroectoderm (NE). The sections were incubated with: A) the polyclonal antiserum against desmin (pDes); B) the polyclonal antiserum against titin (pTitin); C) the monoclonal antiserum BV1118 against vimentin; D) the monoclonal antiserum CK18-2 against cyokeratin 18 and E) the polyclonal antiserum against fibronectin (pFNI). Bars indicate 25 μ m.

Figure 4: Immunofluorescence micrographs of horizontal sections from 8.5 d.p.c. mouse embryos (Theiler stage 12), showing the heart rudiment (HR) and the endocard (Ec). Sections were incubated with A) the polyclonal desmin antiserum (pDes); B) the polyclonal titin antiserum (pTitin); C) the polyclonal vimentin antiserum (pVim), and D) the polyclonal keratin antiserum (pKer). Bar indicates 25 μ m.

drastically reduced in the heart anlage at this stage, although part of the cells still showed a weak punctate to fibrillar staining reaction with the monoclonal keratin antiserum CK18-2 (Fig. 6D). Keratin-positive reactions of differentiating pericardial cells are also observed in the primitive oral epithelium, the thyroid anlage, the lateral plate of the mesoderm and the epidermal ectoderm with the monoclonal keratin antibody (CK18-2). During further differentiation of myocardial cells (e.g., in 9.5 d.p.c. embryos), coexpression of desmin and vimentin is evident. With both antibodies cross-striation can now be observed in individual cells at this stage (Fig. 8A, B) in addition to the cross-striations seen with the titin antibody. Keratin is still weakly expressed in some myocardial cells of 9.5 d.p.c. mouse embryos (results not shown).

Myotome

In the mouse, somites begin to form at Theiler's stage 12 (Theiler, 1972). The somites at this stage are distinctly negative for desmin, titin and keratin, but positive for vimentin. Differentiation of the somite into dermatome, myotome and sclerotome starts at day 9.0 d.p.c. (Theiler stage 14; [7]) and this is accompanied by a positive staining reaction with the desmin and titin antisera in the most cranial myotomes of the embryo (Fig. 9). Desmin staining shows a filamentous staining pattern (Fig. 9A), while titin antibodies give rise to both a punctate and a filamentous staining pattern (Fig. 9B). The vimentin antibodies stain all three somite-derived tissues (dermatome, myotome and sclerotome; Fig. 9C), whereas these tissues are distinctly negative with all the keratin antibodies tested in this study (Fig. 9D).

Figure 5: Immunofluorescence micrographs of frozen sections from 8.5 d.p.c. mouse embryos (Theiler stage 13), showing the tubular heart. Sections were incubated with A) the monoclonal desmin antiserum (DE-B-5); B) the polyclonal titin antiserum (pTitin) and C) the monoclonal vimentin antiserum (BV1118). Bar indicates 50 μ m.

DISCUSSION

Desmin has been described to be a muscle-specific intermediate filament (IF) component in adult vertebrates [14], while titin is a marker in adult striated muscle [50]. Both components may also serve as early markers of the anlagen of these tissues during embryogenesis [43, 44]. In early stages of mammalian myofibrillogenesis, however, the intermediate filament protein (IFP) vimentin is expressed in the developing muscle cells, either without or in combination with desmin. Van Muijen *et al.* [29] and Kuruc and Franke [22] have recently shown that human myocardial cells may even coexpress three different types of IFPs, i.e., keratins, vimentin and desmin [see also: 16].

The main studies, so far, on the expression of muscle-specific components during embryogenesis have concentrated on the chicken system [for review see: 8]. Recently, Tokuyasu and Maher [44] have described the distribution of titin in chicken cardiac premyofibril stages. Hill *et al.* [14] studied the interaction between titin and desmin in postmitotic mononucleated myoblast and concluded that the spatial organization of both components was not coupled. To a much lesser extent, such experiments have been performed in the mouse system [for an example see: 37]. Studies on the formation of IF components during early mouse embryogenesis [9, 17, 18] have shown that no desmin expression can be detected before day 8.0 d.p.c.

In the present study, a punctate staining pattern of titin antibodies was found as the earliest sign of myogenic differentiation in the mouse heart at Theiler's stage 12 (8.25 d.p.c.). Still at the same stage, only a few hours later, desmin expression was also initiated in the heart rudiment, showing a filamentous staining pattern, while titin still showed a punctate staining pattern. Again a few hours later when the heart changed to a tubular organization at Theiler's stage 13, the titin pattern changed to a fibrillar intracellular distribution with the desmin-staining pattern remaining the same as in the foregoing stage. Striation of titin became apparent in the segmented heart at 9.0 d.p.c. (Theiler stage 14). A few hours later at 9.5 d.p.c., but still the same stage according to Theiler, cross-striation of desmin and vimentin was observed. Myogenesis in somites (i.e., in the myotome) was similarly accompanied by a punctate titin staining pattern (Theiler stage

Figure 6: Immunofluorescence micrographs of frozen sections from 9.0 d.p.c. mouse embryos (Theiler stage 14), showing the neural tube (NT), thyroid anlage (TA), ventricular compartments (V), atrial compartments (A), and endocard (asterisks), incubated with A) the polyclonal desmin antiserum (pDes); B) the polyclonal titin antiserum (pTitin); C) the monoclonal vimentin antiserum (BV1118); D) the monoclonal keratin 18 antiserum (CK18-2). Bar indicates 100 μ m.

Figure 7: Immunofluorescence micrographs of a double staining of embryonic mouse myocardium (Theiler stage 14, 9.0 d.p.c.), using A) a monoclonal desmin antibody (DE-R-11) and B) the polyclonal titin antibody (pTitin). Note the cross-striation in the titin-staining reaction (arrow in B) and lack of obvious cross-striation at corresponding sites in the desmin staining (A). Bar indicates 6 μ m.

Figure 8: Immunofluorescence micrographs of frozen sections through the heart of a 9.5 d.p.c. mouse embryo (Theiler stage 14). Sections were incubated with A) the polyclonal desmin antiserum (pDes) and B) the polyclonal vimentin antiserum (pVim). Arrows show cross-striations in myocardial cells. Bar indicates 9 μ m.

14) which was followed by a change to a fibrillar-staining pattern. But here a delay between the appearance of titin and desmin could not be detected, possibly due to the speed of differentiation in myotome cells. To our knowledge, this is the first description of the initiation of desmin expression in the vertebrate heart. Later stages of heart development have been described by Kuruc and Franke [22] in several species. Titin expression, however, has been described in the early stages of chick heart development by Tokuyasu *et al.* [44], who also found a punctate staining pattern in the first stages. During further differentiation, this pattern changes into a fibrillar staining reaction.

In the underlying study, it became also evident that the first expression of mouse desmin could be noted in the neuroectoderm of 8.25 d.p.c. mouse embryos. At 8.5 d.p.c. and later stages, the neuroectodermal layer was negative for desmin.

Coexpression of different types of IFP is a common feature in developing embryonic tissues, and to some extent also in certain adult organs [46-48]. Recently, Van Muyen *et al.* [29] and Gown *et al.* [12] demonstrated a triple expression of keratins, vimentin and desmin in human fetal heart muscle cells. In mouse embryos, vimentin and keratin 18 were coexpressed in differentiating myocard together with titin at first, and later also with titin and desmin. The endocardial cells were always stained by the vimentin antibodies only. Desmin and keratin coexpression was also found to be a transient feature in heart development [22]. At 9.0 d.p.c., keratin reactivity in the myocard was drastically decreased, resulting in a negative reaction in 9.5 d.p.c. mouse embryos. However, at this stage, vimentin and desmin are still coexpressed and even colocalized as concluded from their cross-striated immunofluorescence pattern.

Although *in vitro* studies of myogenesis show that desmin is a candidate for the first sign of myogenic differentiation, our studies clearly show that, *in vivo*, titin, and not desmin, is the first muscle-specific protein to be expressed in presumptive myogenic cells [14, 37]. The titin antiserum used in this study showed a specific

Figure 9: Immunofluorescence micrographs of frozen sections of the myotome of a 9.5 d.p.c. mouse embryo (Theiler stage 14). Sections were incubated with A) a polyclonal desmin antiserum (pDes); B) a polyclonal titin antiserum (pTitin); C) a monoclonal vimentin antiserum (BV1118); and D) a polyclonal keratin antiserum (pKer). (ep: epidermal anlage; d: dermatome; m: myotome; s: sclerotome). Bar indicates 20 μ m.

and exclusive immunoreactivity in the heart anlage of the mouse embryo before desmin expression could be detected in this region, at 8.25 d.p.c. (Theiler stage 12). The typical punctate feature of this labelling pattern, which is comparable to that described by Tokuyasu and Maher [44] for early stages of cardiac myofibrillogenesis in chick embryos, supports the assumption that, at this stage, the antiserum does indeed react with titin.

Unfortunately, no immunochemical proof can be obtained for this observation, since the concentration of the antigen in 8.25 d.p.c. embryos is far too low to allow its detection in immunoblotting studies. At stage 8.5 d.p.c., titin is still present as spots in the developing myocard, while desmin can now clearly be detected and occurs in a filamentous fashion inside these premyocardial cells. Desmin and titin were coexpressed in 9.0 d.p.c. embryonic heart and myotome and showed similarly strong intensities in the immunofluorescence assays on frozen sections of this tissue. At this stage, however, a number of cells showed a striated titin staining pattern, while we could not observe such an organization for desmin. At 9.5 d.p.c., desmin, titin and vimentin were found to be colocalized in these cross-striations. Although it should be kept in mind that the studies so far have been performed in different systems, our observations that titin expression anticipates desmin synthesis seem in contrast with the *in vitro* findings of Hill *et al.* [14]. These authors showed desmin expression in presumptive replicating myoblasts present in embryonic chick skeletal muscle cultures. They could not detect titin until the postmitotic mononucleated myoblast stage and therefore suggested that this constituent is expressed later than desmin in the course of skeletal muscle myofibrillogenesis. Apparently, desmin and titin expression are differently coordinated *in vivo* and *in vitro*.

As far as the myotome is concerned, the present study shows almost simultaneous appearance of titin and desmin in nascent myotome cells. This establishes titin as an early myogenic marker in the myotome in addition to desmin which was hitherto thought to be the earliest marker of myotomal differentiation [20, 40, 49]. Furthermore, the characteristic developmental change of the intracellular distribution of titin from punctate to fibrillar is seen in the myotome as well as in the heart, suggesting similar sequences of early myogenic differentiation operating both organs.

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CHAPTER 2

BABY HAMSTER KIDNEY (BHK-21/C13) CELLS CAN EXPRESS STRIATED MUSCLE TYPE PROTEINS

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ABSTRACT

When Baby Hamster Kidney (BHK-21/C13) cell lines are subjected to low-serum medium, cell morphology changes from polygonal to elongated, while occasionally fusion of cells is also observed. BHK-21 cells initially growing in Eagle's modified minimum essential medium (EMEM) containing 10% newborn bovine serum, were induced to differentiate by changing the culture medium after the cells had grown to confluency. After this point the cells were grown in a low-serum medium (EMEM with 2% normal horse serum), for at least 4 days. The expression of different muscle-specific proteins (desmin, titin and skeletal muscle myosin) and of tropomyosins was studied in both polygonal and elongated BHK-21 cells using the indirect immunofluorescence assay, two-dimensional (2D)-gel electrophoresis and immunoblotting.

Filamentous staining was found with the desmin antisera in the polygonal cells and at all stages of BHK cell elongation. While no reaction was seen with the titin and myosin antibodies in the polygonal cells, a punctate staining reaction for titin was detected 2 days after medium-change, although the cells had not yet elongated. After 4 days titin was found in a striated pattern. Filamentous staining was seen with the skeletal muscle-specific myosin antibody at this stage. Confirmatory results were obtained from immunoblotting assays and 2D-gel electrophoresis of cytoskeletal preparations from undifferentiated and differentiated BHK cells. These latter experiments showed the initiation of tropomyosin expression only in the differentiated cells. The positive staining with antibodies to skeletal muscle myosin and titin indicates a striated-muscle nature of the (elongated) BHK-21/C13 cells.

INTRODUCTION

During *in vivo* and *in vitro* myogenesis of smooth, skeletal and cardiac muscle cells, characteristic muscle-specific markers appear at different stages of differentiation [3, 10, 12, 23, 25, 44, 58]. The muscle-specific intermediate filament protein (IFP) desmin has so far been found in smooth, skeletal and cardiac muscle [7, 30], although some exceptions to this rule have been described [14, 36]. Titin, a high molecular weight protein, is expressed only in striated muscle cell types, both *in vivo* and *in vitro* [10, 22, 25, 35, 57, 58]. It is one of the earliest markers of myofibrillogenesis [12, 44, 53]. Myosin is expressed during a later stage of muscle development, while different isoforms of myosin have been demonstrated in embryonic and adult striated muscles in both birds and mammals [3, 18, 34]. Titin is first expressed in a punctate pattern [6, 44, 53], and in later stages of muscle cell development it shows a striated pattern in both skeletal and cardiac muscle [12, 25, 44]. Upon progression of differentiation or development, desmin and titin are coexpressed, while even later skeletal muscle actin and skeletal muscle myosin heavy chains become detectable in the striated myocytes.

The expression of IFP in BHK-21 cells has been studied by several investigators [2, 13, 15, 40, 47-50, 56]. The two major cytoplasmic IF polypeptides that can be isolated from BHK-21 cells are characterized as desmin and vimentin. So far, the expression of other muscle-specific constituents such as myosin, actin and titin in BHK-21 cells has been studied only sporadically [8, 26, 27].

The detection of desmin in these cells has raised questions concerning their nature [1, 9, 52] and suggests a smooth muscle cell-type of origin. In this study we have used antibodies specific for striated-muscle constituents (titin and skeletal muscle myosin) in combination with more general muscle cell markers (desmin, actin, vimentin) in immunofluorescence and in immunoblotting assays of BHK-21 cells. When these cells were induced to elongate and fuse upon culturing in low-serum medium, profound changes in the expression and organization of these proteins were observed.

MATERIALS AND METHODS

BHK-21/C13 cell cultures

The establishment and characteristics of the BHK-21/C13 cell line have been described previously [8, 11, 13, 26, 27, 30, 32, 33, 40, 49, 51]. BHK-21 cell lines were obtained from three different sources i.e., from Flow Laboratories (Irvine, UK), the Department of Pathology of the University Hospital Nijmegen and the Department of Biochemistry of the University of Nijmegen. These three cell lines were independently cultured in Eagle's modified minimum essential medium (EMEM; Flow Laboratories, Irvine, UK) containing 10% newborn calf serum (Gibco, Paisley, UK), 2 mM L-glutamine (Flow Laboratories) and antibiotics (penicillin and streptomycin). The cells were grown on glass coverslips (Menzel Gläser, FRG) in petri dishes ($\varnothing = 100$ mm; Costar, Cambridge, UK) or six-well plates (Costar) in a humidified incubator at 5% CO₂ and 37°C.

Elongation and partial fusion of the cells was initiated by changing the culture medium after the cells had grown to almost complete confluency. At this point the medium was replaced by a low-serum medium i.e., EMEM containing 2% normal horse serum (Flow Laboratories) and the same additions as described above. After "differentiation", the BHK-21 cells were analyzed at different times following the change of medium. The glass coverslips were washed in phosphate-buffered saline (PBS: 137 mM sodium chloride (Merck, Darmstadt, FRG), 13 mM di-sodium hydrogen phosphate dihydrate (Na₂HPO₄·2H₂O; Merck), 3 mM potassium dihydrogen phosphate (KH₂PO₄; Merck), pH 7.4) [24], the cells were fixed by dipping the glass coverslips in cold methanol (Merck; -20°C) for 3 sec followed by acetone (Merck) fixation (dipping, -20°C), and air-dried at room temperature for 10 min. They were stored at -30°C until use.

Metabolic labelling of BHK-21/C13 cell cultures

Both polygonal and elongated BHK-21/C13 cells, growing in plastic culture flasks (Costar) were washed with PBS and cultured for 1 h in methionine-depleted medium (Eagle's modified minimum essential medium, Flow Laboratories) supplemented with glutamine and antibiotics as above. Immediately thereafter the medium was supplemented with 25 μ Ci.ml⁻¹ [³⁵S]methionine (Amersham, UK; 1000 Ci.mmol⁻¹) and 10% dialyzed newborn calf serum for the polygonal cells and 2% dialyzed normal horse serum for the elongated cells. Cells were labelled for 16 h at 37°C.

Indirect immunofluorescence assays

The BHK-21/C13 cells on glass coverslips were fixed as described above. Five micrometer thick cryostat sections, obtained from fresh frozen hamster tissues were fixed for 5 min in methanol at -20°C and for 1 min in acetone at -20°C and air-dried. Alternatively, the sections were treated for 5 min with 0.5% Triton X-100 (BDH Chemicals Ltd., Poole, UK) in PBS, followed by a PBS washing step.

The sections and BHK-21 cells were incubated with the primary antibody for 30 min at room temperature. After washing with PBS, they were incubated for 30 min at room temperature with the appropriate fluorescein isothiocyanate- (FITC) or tetramethylrhodamine isothiocyanate- (TRITC) conjugated secondary antibody (rabbit anti-mouse IgG FITC, rabbit anti-mouse IgM FITC, goat anti-rabbit Ig FITC, all from Nordic Immunology, Tilburg, The Netherlands, and swine anti-rabbit Ig TRITC from DAKOPatts, Glostrup, Denmark), and washed again with PBS. Sections and BHK-21 cells were mounted in Fluorstab (Euro-Diagnostics BV, Apeldoorn, The Netherlands). Slides were examined with a Leitz Microscope EB20 equipped with epifluorescent illumination. Pictures were taken

on a Tmax-film (Kodak Ltd., Hemel Hempstead, UK) with an automatic camera using an ASA setting of 400.

Cell fractionation and gel electrophoresis

BHK-21/C13 cells were harvested from 150 cm² culture flasks by scraping the cells from the bottom of the flask with a rubber policeman. The cells were washed with PBS containing 0.4 mM phenylmethylsulfonyl fluoride (PMSF; Merck), centrifuged for 10 min at 3000xg, and extracted in 0.5% Triton X-100, 5 mM EDTA (Merck), 0.4 mM PMSF and PBS, pH 7.4, essentially as described [4]. After centrifugation (for 10 min at 0°C, 3000xg), and washing with PBS, the cytoskeletal preparation was dissolved by boiling for 4 min in sample buffer [29], containing 2.3% sodium dodecylsulphate (SDS; BDH Chemicals Ltd.).

One-dimensional gel electrophoresis (Mini-Protean II Electrophoresis Cell, Bio-Rad Laboratories, Richmond, CA, USA) was performed in 7.5% polyacrylamide (Bio-Rad Laboratories) slab gels containing 0.1% SDS [29]. The gels were stained with Coomassie Brilliant Blue R250 (Gurr, Hopkins and Williams, Chadwell Heath, Essex, UK) for 30 min at 50°C to compare the amounts of protein per lane [4].

For 2D-gel electrophoresis, isoelectric focussing conditions according to O'Farrell [37] were used, applying a mixture of pH 3-10 and pH 4-6 ampholines (Biolyte 3/10 and Biolyte 4/6, Bio-Rad Laboratories). Electrophoresis was performed for 7,200 Vh. For the second dimension 12% polyacrylamide SDS-gels were used (Protean II Electrophoresis Cell, Bio-Rad Laboratories).

Immunoblotting

For immunoblotting experiments, the electrophoretically separated polypeptides were transferred (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-Rad Laboratories) to a nitrocellulose sheet (Schleicher and Schuell Membrane Filters BA85, Dassel, FRG) by blotting for 1 h at 100V in a cold (4°C) buffer containing 25 mM Tris (Merck), 192 mM glycine (Merck), 0.02% SDS (BDH Chemicals Ltd.) and 20% methanol [55]. The blots were incubated for 60 min with PBS containing 0.05% Tween20 (Sigma Chemical Company, St.Louis, USA). All reagents were diluted in this Tween20/PBS buffer, which was also used for the washing steps. After overnight incubation with the primary antibodies 9D10 (dilution 1:10), RD301 (dilution 1:5) and MF20 (dilution 1:2), the blots were washed three times for 10 min and incubated for 1 h with horseradish peroxidase-conjugated rabbit anti-mouse Ig (DAKOpatts) diluted 1:400. The blots were washed again three times for 10 min in 0.05% Tween20/PBS, once in PBS alone and stained with 4-chloro-1-naphthol (Merck) and 0.12% hydrogen peroxide (Merck). After staining the blots were rinsed for 5 min with tap water.

Antibodies and reagents

The following antibodies were used in this study (summarized in Table 1):

1. A polyclonal antibody (pDes) to chicken gizzard desmin [43, 44].
2. A mouse monoclonal antiserum (RD301) to desmin [39, 44].
3. An affinity-purified polyclonal rabbit antiserum (pVim) to bovine lens vimentin [42].
4. The mouse monoclonal antibody RV203. This antibody, specific for vimentin, was obtained after a fusion of splenic lymphocytes from a female Balb/C mouse with mouse myeloma Sp2/0-Ag14 cells in polyethyleneglycol-4000 (Merck). The mouse had been immunized twice intraperitoneally and thereafter three times intravenously

with approximately 75 μg of purified bovine lens vimentin per immunization. Growth conditions, cloning and testing procedures were essentially as described before for other antibodies [41]. This antiserum of the IgG1 subclass, tested in indirect immunofluorescence and immunoperoxidase assays, stains tissues of mesenchymal origin and gives a filamentous staining pattern in cultured cells such as BHK-21, HeLa and WiDr. No reaction was found in most epithelial tissues, and in cultured cells such as T24, RT4 and MCF7, known to lack vimentin IFP [21]. In immunoblotting assays of cytoskeletal extracts of bovine lens, BHK-21/C13 and HeLa cells this monoclonal antibody reacts exclusively with a 57 kDa protein, which can be characterized as vimentin in 2D-immunoblots.

5. An affinity-purified polyclonal rabbit antiserum to titin, (kind gift from Dr. D. Gassner, University of Bonn, FRG), was used in a 1:25 dilution [17, 44]. When tested in the indirect immunofluorescence assay, this antiserum reacted specifically with striated (skeletal and cardiac) muscle cells. No reaction was found in smooth muscle cells or in non-muscle cells.
 6. A mouse monoclonal antiserum to titin (9D10) [19, 20, 57].
 7. A mouse monoclonal antiserum to striated muscle myosin (MF20). This antiserum reacts with all sarcomeric myosins (adult and embryonic) [3].
- Monoclonal antibodies 9D10 and MF20 were obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, USA, and the Department of Biology, University of Iowa, Iowa City, IA, USA, under contract N01-HD-6-2915 from NICHD.
8. A mouse monoclonal antibody sm-1 (BioMakor, Rehovot, Israel), recognizing exclusively α -smooth muscle actin [45, 59].
 9. A mouse monoclonal antibody sr-1, recognizing exclusively striated muscle α -actin [46], which was a kind gift of Dr. G. Gabbiani (Geneva, Switzerland).
 10. A mouse monoclonal antibody RAc1, directed against actin, was obtained after fusion of spleen cells from a female Balb/C mouse immunized with a mixture of cytoskeletal preparations (extractions in PBS containing 0.5% Triton X-100) of the T24 and A431 cell lines. This monoclonal antibody of the IgM subclass was characterized by immunofluorescence assays [21, 28] and reacts with myoepithelium of human tissues, with human and hamster skeletal muscle cells and with stress fibers in cultured human cells [21].

Other reagents used in this study were Rhodamine-labelled phalloidin (Molecular Probes, Inc., Junction City, USA) which stains F-actin. The 3.3 μM stock solution of Rhodamine-phalloidin was diluted 1:25 in PBS. The nuclei were stained with Hoechst 33258, at a final concentration of 0.1 $\mu\text{g}\cdot\text{ml}^{-1}$ in 22 mM citric acid ($\text{C}_6\text{H}_8\text{O}_7\cdot\text{H}_2\text{O}$; Merck) and 56 mM di-sodium hydrogen phosphate (Na_2HPO_4 ; Merck).

RESULTS

Antibody reactivity patterns in hamster tissues

Before using the antisera on BHK-21/C13 cells, their specificity in hamster tissues, including heart muscle, tongue and skeletal muscle from the hind limb was tested (see Table 1).

The polyclonal and monoclonal antibodies to desmin showed a positive staining reaction in hamster smooth muscle, skeletal muscle and cardiac muscle (Fig. 1A, C, F). Striation with these antisera was seen in skeletal and cardiac muscle. Also with the polyclonal titin antibody a striated pattern was obtained in cardiac muscle and skeletal muscle (Fig. 1E, G, H). The monoclonal antibody to titin (9D10) showed a striated pattern only in the hamster skeletal muscle (Fig. 1B, D), while no reaction was found in hamster heart tissue

Table 1: Specificity of the monoclonal and polyclonal antibodies, and of the Rhodamine-labelled phalloidin reagent used in this study and their reaction pattern in hamster muscle and nonmuscle cells.

Antibody/ reagent	species Ig- subclass	antigen	muscle cell type			connec- tive tissue	Ref.
			smooth	skeletal	cardiac		
pDes	rabbit serum	desmin	+	+	+	-	43, 44
RD301	mouse IgG2b	desmin	-	+	+	-	38
pVim	rabbit serum	vimentin	-	-	-	+	41
RV203	mouse IgG1	vimentin	-	-	-	+	this paper
pTitin	rabbit serum	titin	-	+	+	-	17, 44
9D10	mouse IgM	titin	-	+	-	-	19, 20, 57
MF20	mouse Ig2b	striated muscle myosin	-	+	+	-	3
sm-1	mouse IgG2a	smooth muscle actin	+	-	-	-	45, 59
sr-1	mouse IgM	striated muscle actin	-	-	-	-	46
RAc1	mouse IgM	actin	-	+	+	-	21, 28
Rhodamine- phalloidin			+ *	+	+	-	24

* Diffuse staining pattern

(Fig. 1I). Neither titin antisera stained smooth muscle cells. (Fig. 1B). The monoclonal antibody sm-1 to smooth muscle actin, showed a strong positive reaction in the hamster blood-vessel smooth muscle cells (Fig. 1J), while striated muscle cells were negative with sm-1. The monoclonal antibody sr-1 was not reactive with hamster (muscle) tissues. The monoclonal actin antibody RAc1 showed a striated pattern in hamster cardiac and skeletal muscle (Fig. 1K). No reaction was found with RAc1 in hamster smooth muscle. The connective tissue fibroblasts and endothelial cells of, for example, hamster tongue stained strongly cells in a filamentous fashion with the polyclonal and monoclonal antibodies to vimentin (Fig. 1L, M). No reaction with the vimentin antibodies was seen in smooth muscle, skeletal muscle and cardiac muscle cells. No reaction was seen in epithelial cells with the vimentin antibodies.

Figure 1: Indirect immunofluorescence of hamster muscle tissues incubated with antibodies to desmin, titin, vimentin and smooth muscle actin to test their tissue specificity in this species. A, B) Double immunofluorescence micrographs of hamster esophagus stained with pDes (desmin; A) and 9D10 (titin; B). Note the reaction of desmin in smooth- and striated-muscle (Sm and Str, respectively), and titin positivity only in striated muscle. C, D) Double-immunofluorescence micrographs of hamster skeletal muscle from hind limb, stained with both pDes (C) and 9D10 (D). E) Reaction pattern of hamster skeletal muscle of the hind limb with pTitin. F-J) Hamster heart muscle stained with pDes (F), pTitin (G, H), 9D10 (I) and sm-1 (α -smooth muscle actin; J). Note that the monoclonal titin antibody and sm-1 do not react with hamster heart muscle cells; Bv: blood vessel. K) Reaction pattern of hamster skeletal muscle of the hind limb with RAc1. L, M) Hamster tongue, showing a positive reaction in the connective tissue fibroblasts with antibody pVim (vimentin; L) and RV203 (vimentin; M); Ep: squamous epithelium; Str: striated muscle. Bar indicates 40 μ m for A-D, M; 25 μ m for E, F; 100 μ m for G, I, J, L; 13 μ m for H; 16 μ m for K.

Figure 2: Phase-contrast (A, B) and immunofluorescence micrographs (C-P) of polygonal BHK-21/C13 cells (A, C, E, G, I, K, M, O) and elongated BHK-21 cells grown for 4 days on low-serum medium (B, D, F, H, J, L, N, P), incubated with pDes (C, D), pVim (E, F), RV203 (G, H), MF20 (I, J), sm-1 (K, L), Rac1 (M, N) and Rhodamine-labelled phalloidin (O, P). Bar indicates 75 μ m for A, B; 25 μ m for C-P.

Induction of BHK-21/C13 cell elongation

When subjected to an alteration in culture conditions, i.e., a change from growth medium containing 10% normal bovine serum to a medium containing 2% normal horse serum, all three established BHK-21 cell lines showed cell elongation after 3-4 days (compare Fig. 2A with 2B) in at least 50% of the cells. Hoechst 33258 staining experiments showed round nuclei in the polygonal BHK-21 cells, while the nucleus changed to a more cigar-like shape after the first signs of cell elongation. Hoechst 33258 staining also revealed some multinucleated cells after 4 days of differentiation, most probably as a result of cell fusion.

Antibody reaction patterns in non-elongated BHK-21/C13 cells

In this study we used BHK-21 cell lines from three different sources. The staining patterns of the different polyclonal and monoclonal antibodies in the indirect immunofluorescence assays were virtually identical for all three BHK-21 cell lines. In the polygonal, non-elongated BHK-21 cells (Fig. 2A) filamentous staining was seen with both desmin antisera (Fig. 2C) and with the vimentin antisera (Fig. 2E, G). Rhodamine-labelled phalloidin showed a stress fiber-like pattern in the polygonal BHK-21 cells (Fig. 2O). No reaction was found with pTitin, 9D10, MF20, sm-1, sr-1 and RAc1 (Fig. 2I, M, K, 3A).

Table 2: Expression pattern of muscle-specific proteins in elongating BHK-21 cells.

Antibody/ reagent	Cells in 10% normal bovine serum (polygonal)	Cells 2 days after medium switch to 2% horse serum (polygonal)	Cells 4 days after medium switch to 2% horse serum (elongated)
pDes; RD301; pVim; RV203	Filamentous	Filamentous	Bundles of filaments
pTitin; 9D10	Negative	Punctate pattern in 50% of the cells	Striation in 50% of the cells; remaining cells filamentous pattern
MF20	Negative	Negative	20%-30% of the cells filamentous staining
sm-1	Negative	Negative	1%-5% of the cells filamentous staining
sr-1	Negative	Negative	Negative
RAc1	Negative	Negative	Stress fibers with striation
Rhodamine- Phalloidin	Filamentous	Filamentous	Stress fibers with striation

Antibody reaction patterns in elongated BHK-21/C13 cells

After 2 days of growing in low-serum medium, when BHK-21 cells had not yet elongated, punctate staining was seen with the polyclonal and monoclonal antibodies to titin (Fig.

Figure 3: Indirect immunofluorescence study with the polyclonal titin antiserum (pTitin; A, C, D) and the monoclonal titin antibody (9D10; B, E, F) in elongating BHK-21/C13 cells. A) Polygonal cells. B) Cells on low-serum medium for 2 days. C-F) Elongated cells, grown on low-serum medium for 4 days. Bar indicates 25 μm for A-C, E; 10 μm for D, F.

3B). The reaction patterns obtained with the other muscle-specific antisera were similar to those observed in the cells grown on high-serum medium. After 4 days of growth under low-serum conditions the elongated BHK-21 cells showed a strong reaction with the titin antisera. In some cells a diffuse to filamentous titin reaction pattern was seen (Fig. 3C, D), but in most of the cells clear striation was observed with the titin antibodies (Fig. 3D, E, F), which seemed to be concentrated along stress fibers. With the desmin antisera filamentous staining was seen in some cells, while most cells showed parallel bundles of desmin IFP. No desmin striation was noted (Fig. 2D). A similar staining pattern was seen with the vimentin antisera (Fig. 2F, H), but both the polyclonal and the monoclonal vimentin antibodies showed much weaker staining of elongated cells than of polygonal cells. The monoclonal antibody MF20 to all types of striated-muscle myosin, showed a strong filamentous reaction in 20-30% of the elongated BHK-21 cells (Fig. 2J). No reaction was seen with the sr-1 antibody. A positive reaction was also seen in some (1-5%) of the elongated BHK-21 cells with the sm-1 antiserum (Fig. 2L). Most of these sm-1 positive cells showed a stress fiber-like pattern, as was also observed with the monoclonal antibody to RAc1 to actin and the Rhodamine-labelled phalloidin (Fig. 2N, P). Striation patterns were seen in most of the cells reacting with phalloidin. RAc1 stained virtually all cells in a differentiated cell culture, with the impression of variable striation along stress fibers, but always less evident than with phalloidin.

Immunoblotting of titin, desmin and myosin

The results of the immunoblotting assays using antibodies to titin, desmin and skeletal muscle myosin (Fig. 4) confirmed the immunofluorescence studies. In the polygonal BHK-

Figure 4: Immunoblots of cytoskeletal extracts of polygonal (lanes a, c, e) and elongated (lanes b, d, f) BHK-21/C13 cells. lanes a and b) Incubated only with the monoclonal titin antibody (9D10). lanes c and d) After the titin incubation as shown in a and b, these blots were reincubated with the monoclonal desmin antibody (RD301). lanes e and f) The blots shown in lanes c and d, were again reincubated with the monoclonal skeletal muscle myosin antibody (MF20). Note that titin and myosin were only detected in the elongated cells, while desmin is present in both polygonal and elongated cells. The specificity of antibodies to myosin and titin is obvious from the negative reactions in lanes c and e.

21 cells a positive reaction was found only for desmin (RD301; Fig. 4, lane c). No immunoreactivity was obtained after incubations with the monoclonal antibody to titin (9D10; Fig. 4, lane a) or with the monoclonal antibody to skeletal muscle myosin (MF20; Fig 4., lane e). In cytoskeletal preparations of elongated BHK-21 cells all these three antisera (9D10, RD301 and MF20) showed a specific positive reaction at the expected molecular weight levels of titin, desmin and myosin, respectively.

2D-gel electrophoresis

Cytoskeletal preparations from metabolically [³⁵S]-labelled polygonal and elongated BHK-21/C13 cells, were analyzed by means of 2D-gel electrophoresis, using isoelectric focussing as the first dimension and SDS-containing polyacrylamide gels as the second dimension.

Figure 5 shows a comparison of the-

Figure 5: Autoradiographs of 2D-gel electrophoretic analyses of polygonal (A,B) and elongated (C,D) BHK-21/C13 cells. The films shown in B and D were exposed for a period six times shorter than films A and C, respectively. Arrows indicate proteins that seem to be synthesized only in elongated cells. This is also true for specific tropomyosins (tm) and the myosin 200 kDa protein (m). v: vimentin, d: desmin and a: actin.

se analyses, indicating a number of profound changes in the protein synthesis patterns upon elongation. The most significant proteins, of which the synthesis is initiated or enhanced as a result of differentiation are indicated by arrows in figure 5C and D, and include constituents with electrophoretic mobilities similar to myosin heavy chain (m) and some of the tropomyosins (tm). Next to these, several other, yet unidentified proteins are synthesized only in the elongated cells. It was not possible to obtain a clear separation of the different isoforms of actin.

DISCUSSION

Initial studies on the nature of BHK-21/C13 cells [32, 33, 48-50] suggested they had a fibroblast-like nature. Since several investigators [1, 9, 13, 56] have shown that BHK-21 cell cultures contain desmin, the muscle-specific IFP, they are now generally regarded as smooth muscle cells. The presence of a second type of IFP, i.e., vimentin, did not interfere with this interpretation, since this IFP is also coexpressed with desmin in developing muscle and many adult smooth muscle cells [1, 9, 52]. However, in a recent study [11] Foddy *et al.* suggested an origin of BHK-21/C13 cells from kidney tubular epithelium on basis of their finding that similar lectins occur on the cell surface of BHK cells and those kidney cells. In line with this idea is the finding of Southgate *et al.* [47], who demonstrated the expression of a type II keratin-like component in a fraction of BHK-21 cell cultures. We could not find keratin expression in the three different badges of cell lines used in this study (unpublished results). Taking together the data published so far, it becomes evident that the discussion about the origin of this cell line is not yet settled.

In the present study we have noticed that cultured BHK-21 cells can change their morphology from polygonal to elongated, with some fused cells, upon a switch in their growth medium from 10% bovine serum to 2% horse serum. These changing conditions, normally used to initiate differentiation in cultured skeletal and cardiac muscle cell cultures, were found to have the same effect on three different batches of BHK-21 cells (see Materials and Methods).

In both the polygonal and elongated phenotypes desmin and vimentin were detected. No quantitative change in desmin immunoreactivity was noticed upon elongation, although the desmin IFP arrangement adapted to the elongated cell shape. In contrast, vimentin immunoreactivity was much less pronounced in elongated cells as compared to the polygonal cells, an observation also made in other differentiating muscle cell cultures [16, 38]. The phenomenon may also parallel the down-regulation of vimentin in differentiating muscle cells *in vivo* [50, 53].

Besides antibodies directed against IFP desmin and vimentin, we have applied antibodies to titin to study the type of muscle differentiation these BHK-21 cell cultures exhibit. Titin has been described as a specific marker for striated muscle differentiation [10, 12, 25, 44, 54, 59]. Our present studies in hamster tissues show that the two titin antibodies used in this study specifically recognize striated muscle. However, in hamster only the polyclonal titin antibody reacted also with cardiac muscle cells. The fact that both antibodies did react with the elongated BHK-21 cells strongly indicate that this cell culture is of a striated-muscle phenotype. The reactivity patterns with the striated-muscle-specific myosin antibody MF20 support this assumption. These results are in accord with preliminary studies (P. van de Ven, Department of Cell Biology and Histology, University of Nijmegen, Nijmegen, The Netherlands; Unpublished) in cultures of human skeletal muscle myoblast. These cells contain desmin, but not titin at the undifferentiated stage. When stimulated to elongate, desmin expression remains, while titin synthesis is induced. These findings are also completely in line with data from Hill *et al.* [23], who found titin and myosin expression upon differentiation of desmin-containing chick myoblasts. Also the finding that the smooth muscle-type actin antibody sm-1 does not

react with the polygonal BHK-21 cells and only reacts with a small percentage of the elongated cell population indicated that BHK-21 cells do not represent smooth-muscle cells [1, 9, 13, 15, 49, 52, 56]. The positivity in some of the elongated cells may be explained by the finding of Woodcock-Mitchell *et al.* [59], that actin isoform expression may change under certain physiological and experimental conditions. Antibody RAc1 to actin was shown to react only in hamster striated muscle cells, and not in smooth muscle cells. Again, the striated muscle phenotype of BHK-21 cells was further substantiated by our finding that RAc1 reacted with elongated (differentiated) BHK-21/C13 cells in a more-or-less striated fashion.

A punctate titin staining pattern was found after 2 days of growing under low-serum conditions. Such titin organization has recently been observed in *in vivo* and *in vitro* during early stages of myofibrillogenesis [6, 44, 54]. During embryogenesis it indicates the first appearance of cardiac or skeletal muscle-type of differentiation [12, 44]. It should be kept in mind that in the embryonic development of cardiac muscles, titin is expressed even before desmin [44]. Although in BHK-21 cells desmin is present in both polygonal and elongated phenotypes, titin is still the earliest marker of striated muscle differentiation, and was expressed even before evident cell elongation was seen. After elongation and fusion of the BHK-21 cells, evident striation of titin was found. Skeletal muscle myosin, present in 20-30% of the elongated BHK-21 cells, was found in a filamentous pattern. No striation of myosin was seen in the myosin positive cells. This observation, in combination with the finding that myosin expression (as detected by MF20) occurred later than titin expression, shows that titin is an early indicator of striated muscle differentiation.

In the 2D-gel electrophoretic analyses we have found that during elongation of the BHK-21 cells synthesis of a number of specific proteins is initiated. The appearance of 200 kDa myosin and tropomyosins in the gels of differentiated cells again indicates the muscle phenotype of these cells [5, 10] and partly substantiates our immunohistochemical findings. Future studies will have to reveal the identity of several other, not yet characterized protein components that are found by 2D-gel electrophoresis to be expressed only, or in a significantly higher quantity in differentiated BHK-21 cells.

We conclude that BHK-21 cell cultures may be used to study cell biological aspects of striated muscle cell differentiation. The advantage of BHK-21 cell lines over several other muscle cell lines that can be induced to differentiate is that BHK-21 cells can be kept in continuous cultures. The muscle cell cultures C2C12, L8 and T984, for example, can be used only for limited passage numbers [31, 38]. Using appropriate antibodies in the indirect immunofluorescence assay the sequence of expression and structural organization of some of the striated muscle-specific constituents can now be studied in BHK-21 cell cultures.

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CHAPTER 3

DIFFERENTIATION OF HUMAN SKELETAL MUSCLE CELLS IN CULTURE: MATURATION AS INDICATED BY TITIN AND DESMIN STRIATION.

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ABSTRACT

This report describes a phenotyping study of differentiating human skeletal muscle cells in tissue culture. Satellite cells (adult myoblasts), isolated from biopsy material, showed a proliferative behaviour in high-nutrition medium, but fused to form myotubes when grown in low-nutrition medium. The expression and structural organization of the intermediate filament proteins desmin and vimentin as well as the sarcomeric constituents α -actin, α -actinin, nebulin, myosin and especially titin during myofibrillogenesis *in vitro*, were studied by means of indirect immunofluorescence assays. The proliferating myoblasts contained both desmin and vimentin, α -actinin and the filamentous form of actin. Shortly after the change of medium, expression of titin, sarcomeric myosin and skeletal muscle α -actin was found in mononuclear cells in a diffuse, filamentous (titin, myosin, α -actin) or punctate (titin, myosin) pattern. Four to ten days after the medium change, mature myotubes showed desmin, titin, α -actinin, nebulin, sarcomeric myosin, and actin cross-striations, while vimentin was no longer detected. We conclude that human skeletal muscle cell cultures are an appropriate model system to study the molecular basis of myofibrillogenesis. Especially the presence of desmin in a striated fashion points to a high degree of maturation of the muscle cell cultures.

INTRODUCTION

In striated muscle tissue contractile properties result from a strictly defined organization of functional proteins in the myofibrillar sarcomere [6,7]. Some of these proteins, like actin and myosin, actually drive contraction, while other constituents are known to link contractile proteins to each other (α -actinin, myomesin), or to keep sarcomeres in register by linking myofibrils to each other or to the sarcolemma (desmin) [7]. In this way, the generated contractile momentum within each fiber can be guided to result in coordinated muscle contraction. Two of the most recently discovered myofibrillar constituents are the giant proteins titin [17] and nebulin [35]. The elastic titin filaments are thought to play an important role in preventing striated muscle from over-stretching [18] in regulating thick filament length [37], and in maintaining the alignment of myosin filaments [14], while the inextensible nebulin filaments probably regulate thin filament length [15, 16].

It is obvious that during myofibrillogenesis, the ordering of different sets of proteins should occur very precisely and in an ordered sequence. Several *in vivo* and *in vitro* studies have been performed, in which the expression and localization of specific myofibrillar and intermediate filament proteins are described during several stages of myogenesis in heart and skeletal muscle (reviewed by Fulton and Isaacs, [8]). The conclusion of these authors is that titin and not desmin, as suggested earlier [10], is the most likely candidate for an important organizer during myofibrillogenesis. Several contradictory observations with respect to myofibrillogenesis have been described, while many aspects still remain unclear [8]. We have monitored *in vitro* differentiation of aneurally cultured human skeletal muscle cells, and characterized different steps in this process by using antibodies to the intermediate filament proteins (IFPs) desmin and vimentin, next to the myofibrillar proteins α -actinin, titin, nebulin, myosin and α -actin.

The aim of this study therefore, was to examine to what extent maturation in human skeletal muscle cell cultures proceeds under optimal conditions of growth and differentiation. On the basis of expression and organization of structural constituents we tested whether these cells show a sufficiently high degree of maturation for use in studying human myofibrillogenesis.

MATERIALS AND METHODS

Muscle cell cultures

Satellite cells were isolated from muscle biopsies obtained during reconstructive orthopedic surgery from patients without known muscular disease, using trypsin and collagenase, essentially according to Yasin *et al.* [8] and Benders *et al.* [4]. The obtained cell suspension was filtered (30 μ m nylon mesh) to remove possible intact myofibers, and seeded in $\varnothing$ 35 mm culture dishes (Costar, Cambridge, MA, USA) in 2 ml Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Paisley, UK) containing 20% fetal calf serum (HyClone Laboratories Inc., Logan, UT, USA), 2% chick embryo extract (Flow Laboratories, Irvine, UK), 2 mM glutamine (Gibco BRL), 100 U.ml⁻¹ penicillin (Gibco BRL) and 1 μ g.ml⁻¹ streptomycin (Gibco BRL), and grown to about 80% confluence in a humidified incubator (Heraeus, Hanau, FRG) at 37°C and 5% CO₂. Then cells were trypsinized and reseeded in $\varnothing$ 35 mm culture dishes at a maximal split ratio of 1:6. Cells were frozen in liquid nitrogen in culture medium complemented with 10% DMSO (Merck, Darmstadt, FRG) after trypsinization. For indirect immunofluorescence experiments these cells were thawed quickly, grown to near confluency in $\varnothing$ 35 mm culture dishes, trypsinized and reseeded onto 10 well multispot microscopic slides (Cel-line Associates Inc., Newfield, NJ, USA). After 16 to 24 h the medium as described above, was replaced by an Ultrosor G (Gibco BRL) containing culture medium described by Benders *et al.* (1991), except that the rat brain extract was replaced by human brain extract (HBE: see below). Differentiation of the muscle cells normally occurred upon confluency, but was in most experiments induced before myotubes became visible, by changing the high-nutrition culture medium to a low-nutrition medium (DMEM, 0.4% Ultrosor G, 5% HBE, glutamine and antibiotics) just before the cultures reached confluency. The medium was changed every 3 days. During differentiation, every 3 days half of the medium was replaced by fresh medium. For cell fractionation experiments, cells were cultured in 75 cm² tissue culture flasks (Costar).

Human brain extract (HBE) was prepared by homogenizing normal, adult tissue of the frontal lobe of the cerebral cortex obtained at autopsy 1 h after death, in DMEM, using a Potter-Elvehjem glass-teflon homogenizer and a pestle with a clearance of 0.05 mm. After centrifugation of the homogenate for 1 h at 100,000 $\times$ g, the supernatant was stored at -70°C. Before use, the HBE was sterilized using a 0.2 μ m filter (Schleicher & Schuell, Dassel, FRG). The concentration of HBE in the culture media was equivalent to 10 mg tissue per ml medium.

Muscle tissue specimen for immunohistochemistry

The skeletal muscle tissue used for our immunohistochemical study was obtained at autopsy of a 1-day-old boy without known muscle disorder. The child was born after a pregnancy of almost 28 weeks. Immunohistochemistry was carried out together with routinely performed histopathological staining for diagnostic reason. The tissue was frozen in liquid nitrogen immediately after dissection.

Indirect immunofluorescence assays

Muscle cells cultured on glass slides were briefly rinsed in phosphate-buffered saline (PBS: 137 mM sodium chloride (Merck), 13 mM di-sodium hydrogen phosphate dihydrate (Na₂HPO₄·2H₂O; Merck), 3 mM potassium dihydrogen phosphate (KH₂PO₄; Merck), pH 7.4) and fixed in methanol (Merck; -20°C, 30-60 sec) and acetone (Merck; -20°C, 5-10 sec) at several time intervals following the medium change. When not immediately

tested, the slides were frozen at -25°C after air-drying. Cryostat tissue sections (6 μ m thick) were air-dried and treated for 10 min with 0.5% Triton X-100 in PBS. Before incubation the sections were rinsed in PBS. The cells or tissue sections were incubated with the primary antibody for 30 min at room temperature. After extensive washing for 30 min in PBS they were incubated with fluorescein isothiocyanate- (FITC) conjugated rabbit anti-mouse Ig or swine anti-rabbit Ig (Dakopatts, Glostrup, Denmark). The slides were washed again in PBS and mounted in Gelvatol (Monsanto, St. Louis, MO, USA). Slides were viewed with a Zeiss Axioskop microscope with epifluorescent illumination (Carl Zeiss, Oberkochen, FRG). Pictures were taken using 400ASA TMY film (Kodak, Rochester, NY, USA), using an automatic camera.

Antibodies and reagents

The following monoclonal and polyclonal antibodies were used in this study:

1. 9D10, a mouse monoclonal antibody to titin [36], obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University, School of Medicine, Baltimore, MD, USA, and the Department of Biology, University of Iowa, Iowa City, IA, USA, under contract N01-HD-6-2915 from the NICHD.
2. A mouse monoclonal antibody RV202, specific for vimentin [23].
3. A polyclonal rabbit antiserum (pDes), raised against chicken gizzard desmin [22].
4. An affinity-purified polyclonal rabbit antiserum (pVim), raised against bovine lens vimentin [21].

These antibodies to desmin and vimentin are available from Euro-Diagnostics B.V., Apeldoorn, The Netherlands.

5. A mouse monoclonal antibody V9 to vimentin, purchased from Dakopatts.
6. The mouse monoclonal antibody NB2, specific for nebulin [9], purchased from Sigma Chemical Company, St. Louis, MO, USA.
7. A polyclonal rabbit antiserum raised against α -actinin, which was a kind gift from Dr. B. Jockusch, Bielefeld, FRG.
8. The mouse monoclonal antibody MF20, recognizing all forms of sarcomeric myosin [2], which was a kind gift from Dr. D. Fischman, New York, NY, USA.
9. A mouse monoclonal antibody sr-1, specific for striated muscle α -actin [28], which was a kind gift from Dr. G. Gabbiani, Geneva, Switzerland.
10. Rhodamine-labelled phalloidin (Molecular Probes Inc., Eugene, OR, USA) was used for staining of filamentous actin (F-actin).

Cell fractionation, gel electrophoresis, and immunoblotting

Human skeletal muscle cells, grown in 75 cm² tissue culture flasks as described above, were collected and the cytoskeletal fraction was prepared as described [27]. Polyacrylamide (PAA) gel electrophoresis of cell fractions enriched for cytoskeletal proteins, and staining of the gels, and immunoblotting were carried out essentially as described [27], except that 7% or 10% PAA gels were used with a 3% PAA stacking gel. High- and low-molecular weight markers (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) were used to identify the molecular weight of the separated proteins.

RESULTS

Characterization of antibody reactivity patterns in human skeletal muscle

Figure 1: Immunofluorescence micrographs of neonatal human muscle tissue sections incubated with antibodies to α -actinin (A), desmin (B), titin (C, E), nebulin (D), myosin (F), and vimentin (G), or stained with Rhodamine-labelled phalloidin (H). Bar indicates 10 μm for F; 13 μm for A, B, E; 17 μm for C, D, G; 20 μm for H.

Figure 2: Phase-contrast micrographs of aneurally cultured human skeletal muscle cells. A) Proliferating cells. B) Young myotubes with predominantly centrally located nuclei (arrowheads). C) Mature, contracting myotubes with cross-striations (arrows) and peripherally located nuclei (arrowheads). Bar indicates 100 μm for A and B; 50 μm for C.

Since the specificity of some of the antibodies used was not known for human tissues, the reactivity patterns of the antibodies were established in normal premature human skeletal muscle. Sections of the biopsy material, described above, showed a striated pattern with all the applied antibodies, except for sr-1. The antibodies to α -actinin (Fig. 1A) and desmin (Fig. 1B) showed a discrete striated staining pattern with fluorescence at regular intervals. The titin antibody 9D10 (Fig. 1C, E), the nebulin antibody NB2 (Fig. 1D)

and the myosin antibody MF20 (Fig. 1F) displayed a doublet banding pattern, the first two antibodies showing discrete lines, and the latter staining a somewhat broader region of the sarcomere. RV202, the monoclonal antibody to vimentin, strongly stained blood vessels and interstitial tissue, and also muscle fibers, resulting in a striation, albeit to a much lesser extent (Fig. 1G). This striated pattern was also seen with V9, a second vimentin monoclonal antibody as well as pVim, a polyclonal antiserum to vimentin (not illustrated). Staining with Rhodamine-phalloidin resulted in a reactivity pattern with alternating strong and weak fluorescent lines (Fig. 1H). No clear reactivity was observed with the sr-1 antibody to striated muscle specific α -actin. Except for the vimentin antibodies, the reaction patterns were equal to those in adult human muscle (not illustrated).

Muscle cell cultures

Differentiation of the initial spindle- to polygonal-shaped skeletal muscle cells in culture (Fig. 2A) was initiated when the cultures reached confluence, or when the culture medium was changed to a low-nutrition medium. Within 1 day after the change of medium, the cells started to elongate and fused to form syncytia, often with clusters of nuclei (see below). Subsequently these syncytia matured gradually: long myotubes were formed, nuclei were often translocated from a more central position (Fig. 2B) to the periphery of the myotubes (Fig. 2B, C). Eventually cross-striations visible with phase-contrast optics developed (Fig. 2C).

Proliferating myoblasts

The human skeletal muscle-derived cells, grown in high-nutrition culture medium and fixed before reaching confluence, showed a filamentous staining pattern with the desmin antiserum in more than 95% of the cells (Fig. 3A), and with the vimentin antibody in virtually all cells (Fig. 3B). A periodically interrupted, stress fiber-like reactivity pattern was observed after an incubation with Rhodamine-phalloidin (Fig. 3C) or the antiserum to α -actinin (Fig. 3D). The antibodies to titin, nebulin, myosin and striated muscle α -actin did not stain the myoblasts.

Non-proliferating, elongated mononuclear cells

Shortly (1 to 2 days) after the medium change, when the human skeletal muscle cells had elongated, they were tested for the presence of the proteins recognized by our panel of antibodies and reagents. Longitudinally oriented stress fiber-like structures were stained in a periodically interrupted pattern with the α -actinin antiserum (Fig. 4A) and with Rhodamine-phalloidin (Fig. 4D). A relatively low number of cells showed a similar reactivity with the antibody to striated muscle α -actin, accompanied by a diffuse staining of the cytoplasm (Fig. 4B). The reaction patterns of the antibodies to desmin (Fig. 4C) and vimentin (Fig. 4G) remained filamentous. These filaments, however, were oriented more lengthwise. In addition to longitudinally oriented filaments, the antibody to myosin now also stained cytoplasmic spots, which seemed associated with these filaments, often together with a diffuse cytoplasmic staining (Fig. 4E). The antibody to nebulin was negative in these cells (Fig. 4F). The titin antibody showed a diffuse, punctate or filamentous reactivity. These titin reaction patterns will be described below in more extent. The number of cells expressing muscle-specific proteins varied per experiment and was dependent on the exact moment of fixation after the medium change.

Figure 3: Immunofluorescence micrographs of proliferating human skeletal muscle satellite cells incubated with the antibodies to desmin (A), vimentin (B) and α -actinin (D) or stained with Rhodamine-labelled phalloidin (C). Bar indicates 75 μ m.

Mature myotubes

When the human skeletal muscle cells were cultured in low-nutrition medium for 4 to 10 days, the cultures contained numerous long myotubes consisting of fused myoblasts and containing up to 50 nuclei. Most of these myotubes showed a cross-striated pattern with the polyclonal antibodies to α -actinin (Fig. 4H). Such a banding pattern was also observed in a majority of the myotubes when F-actin was stained with Rhodamine-phalloidin (Fig. 4K). The monoclonal antibodies to myosin (Fig. 4L), nebulin (Fig. 4M), and titin (see below) now also detected a typical cross-striated distribution pattern for these constituents. Desmin cross-striation was also seen at this stage of myotube differentiation (Fig. 4J), although less frequently than for the other muscle-specific proteins. The antibodies against myosin, titin and nebulin revealed regularly arranged doublet banding patterns, while the antibodies to α -actinin and desmin as well as the Rhodamine-phalloidin reagent stained discrete lines with a relatively constant distance. The antibody to striated muscle α -actin showed a staining pattern comparable with that in mononuclear cells. Longitudinally oriented fibrils were stained, while a diffuse reactivity in the cytoplasm was also seen (Fig. 4I). The expression of vimentin as seen with antibody RV202, was dramatically decreased in mature myotubes (Fig. 4N; arrows).

Titin distribution during several stages of human muscle cell differentiation in culture

Proliferating human myoblasts were negative for 9D10, the monoclonal antibody to titin. Soon after the change to low-nutrition culture medium, isolated cells that had become

Figure 4: Immunofluorescence micrographs of postmitotic mononucleated myoblasts after change to low-nutrition medium (A-G) and of mature, fused myotubes (H-N) incubated with the antibodies to α -actinin (A, H), striated muscle-specific α -actin (B, I), desmin (C, J), myosin (E, L), nebulin (F, M), and vimentin (G, N), or stained with Rhodamine-labelled phalloidin (D, K). Note the vimentin-negative myotube in N (arrows). Bar indicates 50 μ m for A-M; 25 μ m for N.

Figure 5: Immunofluorescence micrographs of different stages of differentiating muscle cells (see text), incubated with the antibody to titin. Bar indicates 50 μm for A, B, D, F, G, H; 40 μm for C, E.

spindle shaped but not yet elongated, showed a diffuse staining pattern including a punctate titin localization in close proximity to the nucleus (Fig. 5A). In cells aligning prior to fusion, the same reactivity patterns were observed. However, the localization of the spots was more uniformly spread throughout the cytoplasm (Fig. 5B). Following cell-fusion these seemed to associate with longitudinally oriented fibrils (Fig. 5C), most probably the stress fibers. A filamentous, stress fiber-like titin reaction pattern was visible in somewhat further differentiated polynuclear cells (Fig. 5D, E). The latter two reactivity patterns were also observed in some elongated mononuclear cells. Subsequently, short stretches of myofibrils developed showing the typical striated doublet staining pattern of titin (Fig. 5F). During further differentiation and growth the number of myofibrils in the myotubes increased (Fig. 5G), aligning and filling most of the cytoplasm, resulting in a reaction pattern more or less comparable with mature myofibers in tissue sections (Fig. 5H).

Polyacrylamide gel electrophoresis

Fractions enriched for cytoskeletal proteins from proliferating human satellite cell cultures and from cultures showing large numbers of matured myotubes, were fractionated on 10% polyacrylamide SDS-gels. Specific proteins could be identified by their well-characterized molecular weights. Cells from proliferating cultures contained relatively small amounts of α -actinin, desmin and actin, while vimentin was present in large quantities (Fig. 6, lane A). Cultures that were differentiated for 6 or 7 days, showed an increased expression of α -actinin and actin. The vimentin:desmin ratio was dramatically decreased, while myosin and tropomyosin were only found in detectable amounts in differentiated cells (Fig. 6, lane B). However, unidentified proteins, exclusively expressed in differentiated cells, were observed within the stacking gel (not illustrated). Considering that the molecular weights of these proteins exceeded that of myosin by far, we expect them to represent titin and nebulin.

Table 1: Expression pattern of muscle differentiation markers during human myofibrillogenesis.

Antibody/ reagent	Antigen	Proliferating myoblast	Non-proliferating, elongated myoblasts	Matured myotubes
Polyclonal α - actinin	α -Actinin	Stress fiber-like	Stress fiber-like	Cross-striated
sr-1	Striated muscle specific α -actin	Negative	Diffuse/ stress fiber-like	Diffuse/ stress fiber-like
pDes	Desmin	Filamentous	Filamentous	Cross-striated
Rhodamine- Phalloidin	F-actin	Stress fiber-like	Stress fiber-like	Cross-striated
MF20	Sarcomeric myosin	Negative	Diffuse/punctate/ filamentous	Cross-striated
NB2	Nebulin	Negative	Negative	Cross-striated
RV202	Vimentin	Filamentous	Filamentous	Negative
9D10	Titin	Negative	Diffuse/punctate/ filamentous	Cross-striated

Figure 6: Gel electrophoresis (A, B) and immunoblotting (C-G) of cytoskeleton-enriched fractions of proliferating (lane A, lanes Cp-Gp) or differentiated (lane B, lanes Cd-Gd) human skeletal muscle cells. The positions of molecular weight markers for A and B are indicated on the left, and for lanes C-G on the right. Arrowheads point to cytoskeletal proteins identifiable by their molecular weights. M: myosin heavy chain, 200 kDa; A1: α -actinin, 100 kDa; V: vimentin, 57 kDa; D: desmin, 53 kDa; A2: actin, 43 kDa; T: tropomyosin, 36 kDa. Fractionated proteins transferred to nitrocellulose were incubated with the antibodies to sarcomeric myosin (C), α -actinin (D), vimentin (E), desmin (F), or striated muscle-specific α -actin (G).

Immunoblotting

Our results from immunofluorescence studies were confirmed by immunoblotting assays using antibodies to myosin, α -actinin, vimentin, desmin, and striated muscle α -actin. Sarcomeric myosin (Fig. 6C), as well as striated muscle α -actin (Fig. 6G) were exclusively detected in differentiated cultures, while an incubation with the antibodies to α -actinin (Fig. 6D) and desmin (Fig. 6F) resulted in significantly stronger reactions in the lanes containing differentiated cells compared to the lanes containing proliferating cells. Vimentin was detected in equal amounts in both stages of differentiation.

DISCUSSION

In this study we have attempted to answer two main questions: 1) What final stage of differentiation can non-innervated human skeletal muscle cells reach in culture under optimal conditions? This matter was approached by using the expression and organization of certain muscle-specific constituents as markers for certain stages of muscle cell differentiation. 2) How is titin organized during the different stages of *in vitro* muscle cell differentiation? In particular we were interested in the very first signs of titin expression, its organization at that stage and the further rearrangements up to the fully striated organization.

Most muscle-specific proteins are absent in proliferating satellite cells

Proliferating satellite cells were stained by the antiserum to desmin, as might be expected from the fact that desmin stains activated satellite cells *in vivo* [12]. This result indicates that our cultures contained mainly muscle cells. Desmin-negative cells probably represented fibroblastic cells. The presence of desmin as well as vimentin in the muscle cells is comparable to the *in vivo* situation where during early stages of myogenesis these IFPs are coexpressed in muscle cells [3, 25]. The initiation of desmin synthesis in proliferating mouse and rat myoblasts *in vitro* has been described previously [1,13]. Immunofluorescence assays and gelelectrophoresis- and immunoblotting-experiments showed that striated muscle-specific α -actin, titin, nebulin, and sarcomeric myosin were absent in these cells.

Muscle-specific protein expression

After elongation of the cultured human skeletal muscle cells they remained desmin- and vimentin-positive, although the IFPs changed to a more longitudinally oriented pattern, as was also seen in "differentiating" BHK-21/C13 cells [27]. In contrast to titin, sarcomeric myosin and striated muscle-specific α -actin, nebulin expression was not found in these cells, indicating once more that during myogenesis not all myofibrillar proteins are coexpressed at all stages of differentiation *in vitro*. At this stage and more differentiated stages, we never observed cytoplasmic spots with any antibody except for the titin and myosin antibodies. This indicates that during human myofibrillogenesis, I-Z-I complexes of titin and α -actinin and A-band complexes consisting of myosin and associated proteins [6] do not develop from these spots as observed in chicken skeletal muscle cultures [30, 31].

A high degree of maturation of the muscle cell cultures is indicated by desmin cross-striation

Maturity of the human muscle cell cultures was tested by incubation of the cultures after 4 to 10 days following the change to low-nutrition medium. The process of down-regulation of vimentin paralleled by an upregulation of desmin as the main IFP *in vivo* [3, 32] is mimicked *in vitro*. In the most mature myotubes no vimentin was detected, while desmin showed a cross-striation in varying numbers of myotubes. However, in our cultures no vimentin cross-striation was observed at any developmental stage (see below). The antibodies to α -actinin, myosin, nebulin and titin, as well as the Rhodamine-phalloidin all showed cross-striations. The fact that desmin organized in cross-striations, which was reported earlier to occur only in human skeletal muscle cell cultures when co-cultured with neural tissue [19], indicated a high degree of maturity of the myotubes. The altered desmin:vimentin ratio and the upregulation of several muscle proteins could be confirmed by gel electrophoresis and immunoblotting experiments.

Titin expression during human myofibrillogenesis

In low-nutrition medium the mononuclear muscle cells alter their morphology. The spindle-shaped or polygonal cells tend to elongate before fusion and further differentiation. Besides desmin, titin was the only muscle-specific protein detected in non-elongated cells in a diffuse to punctate pattern. These results confirm observations in skeletal muscle cell cultures derived from other species [5, 13] and *in vivo* studies with embryos from mice [26] and rabbits [33]. Titin is one of the first typical myofibrillar proteins expressed in

differentiating muscle cells. During maturation the spot-like titin reactivity, indicating titin aggregates, changed from a random distribution to a localization associated with fibrils, probably stress fiber-like structures. The latter structures are present during this stage of development as seen with the reagents detecting filamentous actin and the antibodies to α -actinin. Subsequently, the antibodies against titin stained longitudinal fibrils which eventually changed to myofibrils, showing the typical striated titin doublets. After 4 to 10 days in low-nutrition medium, the cultures were occupied by numerous myotubes, showing a mature morphology when stained with the 9D10 titin antibody.

Vimentin as constituent of the Z-line region in immature human skeletal muscle

In our experiments we used autopsy material from a prematurely born boy, to characterize antibodies and reagents for their reactivity patterns in human skeletal muscle. The antibodies to desmin and α -actinin stained the Z-line region. The titin and nebulin antibodies are known to recognize an epitope around the A-I junction [36] and epitopes within the I-band [16], respectively. Consequently, doublets of distinct striations were seen. MF20, the antibody to sarcomeric myosin stained A-bands, which alternate with myosin-negative M-lines. Rhodamine-phalloidin stained alternating weak and strong bands, probably representing the thin filaments and the actin filaments beneath the sarcolemma at the level of the M-line, respectively [7]. Vimentin is normally present in tissues of mesenchymal origin. As a result, our antibodies to vimentin stained vascular endothelium and interstitial fibroblasts. However, an obvious and significant cross-striated staining reaction was seen in muscle fibers of the immature skeletal muscle. From these findings we can conclude that during human myogenesis vimentin is colocalized with desmin in the Z-line region at least up to 28 weeks of gestation, as was demonstrated also in avian skeletal muscle [11]. As already described by Sarnat *et al.* [25] and Barbet *et al.* [3], in our hands normal, non-regenerating adult human muscle fibers did not contain detectable amounts of vimentin, while regenerating fibers showed a diffuse vimentin staining pattern. The presence of vimentin in immature (embryonic) human muscle has been shown previously, in a cross-striated fashion, but was never discussed [3]. The absence of vimentin cross-striations in our cultures might be explained by the fact that regeneration, and not embryogenesis, is mimicked in our experiments.

We conclude that cell cultures of human skeletal muscle are an appropriate tool to study virtually all developmental stages of myofibrillogenesis. Desmin cross-striation was described for the first time in aneurally cultured human myotubes, indicating that the culture medium we used enhances myofibrillogenesis during differentiation. Although cell cultures of human skeletal muscle were per se not always very successful as a model system for studying muscular diseases [20], we feel that these cultures might be useful to study pathogenesis of muscular diseases which are characterized by, or even caused by an abnormal distribution of specific sarcomeric or cytoskeletal constituents [24, 29, 34].

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CHAPTER 4

CHARACTERIZATION OF CARDIOTIN, A STRUCTURAL COMPONENT IN THE MYOCARD

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ABSTRACT

The characterization and subcellular distribution of cardiotin, a structural component of striated muscle, is described using a monoclonal antibody. This high molecular mass component (> 300 kDa) is expressed in the myocard of several species and to a lesser extent also in skeletal muscle. Cardiotin is not found in smooth muscle tissues, other mesenchymal or epithelial tissues. The cardiotin distribution pattern is independent of other sarcomeric components, such as desmin, myosin, actin, titin, nebulin, and desmoplakin, and shows a longitudinal filamentous localization between myofibrils. The average distance between parallel running cardiotin filaments is approximately $2.3 \mu\text{m}$, as concluded from confocal scanning laser microscopic analysis of double-immunolabelled muscle preparations. The cardiotin filamentous staining reaction is oriented perpendicularly to the typical cross-striations observed with antibodies to desmin, spanning several sarcomeres and showing a length between 12 to $80 \mu\text{m}$ in frozen sections. Its localization pattern suggests a possible link with the sarcoplasmic reticulum. We have never observed cardiotin filaments to cross the intercalated discs, stained by antibodies to desmoplakins or desmin. Cardiotin cannot be solubilized from cardiac muscle by nonionic detergents or high concentrations of KCl or KI, suggesting a structural role in the myocard. The protein could so far not be detected in developing embryonic heart, but expression seems to be initiated after birth, depending on the species examined.

INTRODUCTION

The expression of specific proteins can distinguish the different stages of muscle cell differentiation during myofibrillogenesis and cardiogenesis *in vivo* and *in vitro* [2, 6, 13, 19, 21, 44, 45, 55, 56, 63]. Over the past two decades many of such specific structural muscle components and their functions have been described [For reviews see: 1, 4, 5, 54].

Amongst these constituents are the intermediate filament proteins desmin and vimentin, which are inversely regulated in both smooth muscle cells and striated muscle cells [3, 29, 49]. In adult striated muscle desmin is found in the Z-line [17] where it maintains the proper alignment of myofibrils during contraction and relaxation [6, 30]. Vimentin is normally found in early stages of muscle cell differentiation and can coexist or even copolymerize with desmin [10, 39, 48, 51]. At certain stages of development of the heart of the mouse and the rabbit even cross-striations of vimentin have been described [44, 55, 58]. Vimentin is also found in a cross-striated pattern in human cultured muscle cell lines [56]. During further stages of myotube formation, vimentin expression is down-regulated both *in vitro* and *in vivo* [14, 38, 44, 56].

Different isoforms of actin, myosin and tropomyosin are expressed during muscle cell development [1, 2, 6, 31, 32, 63, 65]. Until now the functional significance of expressing such different protein isoforms during different stages of muscle development is not clearly understood [1].

A protein only found in skeletal and cardiac muscle cells is titin, with an extremely high molecular mass of 3.10^6 Dalton [20, 59]. Titin, also known as connectin [35], is localized between the M- and Z-lines [6, 12, 13, 66] and is assembled in its mature pattern earlier than other major contractile proteins [19, 44, 45, 52, 55]. According to Whiting *et al.* [66] and Trinick [54] a part of the titin sequence, i.e., the part associated with the Z-line, has elastic properties, thus preventing the sarcomere from overstretching. The other region of the molecule seems to regulate the assembly of myosin and other thick filament proteins [22, 54].

Another giant muscle protein is nebulin [60] with a molecular mass of approximately 8.10^5 Dalton [50, 54]. Nebulin, only found in skeletal muscle and not in heart muscle or smooth muscle cells, is an inextensible filamentous protein [61] that appears to be

associated with the thin filaments, but not with titin [36, 61], and has been suggested to regulate actin assembly [23, 25, 26].

Only recently the biogenesis and organization of the membrane systems in striated muscle cells have received renewed attention. In particular studies on the regulation of excitation-contraction coupling upon differentiation of the muscle cells have led to the generation of several probes for proteins involved in this process [7, 8].

We have detected a new structural muscle cell constituent with a high molecular weight, and a specific distribution pattern in striated muscle, particularly in the myocard. It was characterized by a monoclonal antibody [46] and designated cardiotin. Here we describe the tissue and species distribution patterns of cardiotin, its subcellular localization in muscle cells and its relation to other muscle cell components.

MATERIALS AND METHODS

Tissue distribution

Normal adult human tissues obtained by autopsy, i.e., heart (ventricle and atrium, both right and left; apex), skeletal muscle, tongue, esophagus, stomach, small intestine, colon, liver, bile bladder, kidney, urine bladder, prostate, ovary, uterus, breast, lung, pancreas, lymph follicle, spleen, and brain were immediately frozen in liquid nitrogen (N_2).

Heart tissue of a 16-week-old human embryo was obtained during autopsy and immediately frozen in liquid N_2 .

From different animals i.e., *Xenopus laevis*, chicken, mouse, rat, hamster, rabbit, cat, dog, goat, cow, and Rhesus monkey, heart tissue (myocard), tongue, small intestine, and skeletal muscle were stored in liquid N_2 .

All the tissues were mounted in Tissue-Tek (OCT-compound; Miles Inc., Elkhart, IN, USA) and 5 μ m sections were cut at -25°C and air-dried overnight at room temperature.

Antibodies

Antibodies used in this study are:

1. The monoclonal R2G antibody to cardiotin (IgM), obtained as follows: chicken gizzard desmin (for purification method see [41, 42]) was used for the immunization. A female Balb/C mouse was immunized twice intraperitoneally and three times intravenously with approximately 75 μ g purified chicken gizzard desmin in 250 μ l phosphate-buffered saline (PBS: 137 mM sodium chloride (NaCl; Merck, Darmstadt, FRG), 13 mM di-sodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$; Merck), 3 mM potassium dihydrogen phosphate (KH_2PO_4 ; Merck), pH 7.4). After the immunization period (3 weeks), the mouse was killed, and spleen cells were fused with Sp2/0-Ag14 myeloma cells, cloned and grown according standard protocols [24, 45]. The monoclonal antibody R2G was selected on basis of its strong reactivity pattern with human cardiac muscle, while other cell types were not stained, except for a relatively weak reaction with skeletal muscle.
2. A mouse monoclonal antibody (RD301) to desmin [40, 44].
3. A mouse monoclonal antibody (RV203) to vimentin [45].
4. Dp1&2-2.15, a mouse monoclonal antibody to desmoplakin (Progen Biotechnik GmbH, Heidelberg, FRG) [11].
5. DE-R-11, a mouse monoclonal antibody to desmin (DAKOpatts, Glostrup, Denmark) [3].
6. A polyclonal rabbit antiserum to chicken gizzard desmin (pDes) [43].
7. An affinity-purified polyclonal rabbit antiserum to titin (kind gift from Dr. D. Gassner, Bonn, FRG) [15, 44].

8. A mouse monoclonal antibody (9D10) to titin [18, 62].
- Both antibodies to titin gave exactly the same staining patterns when tested on human striated muscle tissues. Both are negative in non-striated muscle tissue.
9. A mouse monoclonal antibody (MF20) to sarcomeric (embryonic and adult) myosin [2].
 10. M3F7, a mouse monoclonal antibody to type IV collagen [9].
- Monoclonal antibodies 9D10, MF20, and M3F7 were obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, University of Iowa, Iowa City, IA, USA, under contract N01-HD-6-2915 from the NICHD.

Immunocytochemistry

In this study indirect immunofluorescence assays were performed. In all cases 5 μ m thick cryostat sections, obtained from freshly frozen tissues were used. The sections were treated routinely for 5 min with 0.5% Triton X-100 (BDH Chemicals Ltd., Poole, UK) in PBS, followed by a PBS washing step for 5 min.

Alternative pretreatment of the tissue sections with 0.5% Triton X-100 in PBS containing either 0.6 M potassium iodide (KI; Merck) and 1 M potassium chloride (KCl; Merck), or with 0.5% sodium deoxycholate (NaDOC; Merck) and 1% Tween40 (Sigma Chem. Co, St. Louis, MO, USA) was applied for 5 min, followed by a PBS washing step for 5 min.

Parallel fresh cryostat sections were directly fixed for 5 min in methanol (-20°C; Merck), then for 1 min in acetone (-20°C; Merck) and air-dried.

Tissue sections (see: Tissue distribution) of different species (i.e., *Xenopus laevis*, chicken, mouse, rat, rabbit, hamster, cat, dog, goat, cow, Rhesus monkey and man), pretreated with 0.5% Triton X-100 were incubated with the primary antibody for 30 min at room temperature, followed by a washing step with PBS. For the second incubation step the appropriate fluorescein isothiocyanate- (FITC) conjugated antibody (goat anti-mouse IgG or goat anti-mouse IgM; both from Southern Biotechnology Associates (SBA) Inc., Birmingham, Alabama, USA) were used. For double-labelling studies Texas Red- (TR) conjugated antibodies (goat anti-mouse IgM or goat anti-mouse IgG1; SBA) were used. The secondary antibodies were applied for 30 min at room temperature. After three washing steps with PBS, the immunofluorescent-stained tissues were mounted in Mowiol (Hoechst, Frankfurt, FRG) [37].

Proliferating and differentiating human skeletal muscle cell cultures were grown on glass coverslips (for method see: [56]). Differentiation of the human skeletal muscle cells was initiated by a medium-switch from a high-nutrition medium to a low-nutrition medium [45, 56].

Cytospins were made from isolated rabbit cardiomyocytes (for method see: [57]) in PBS containing 20% fetal calf serum (5 min, 800 rpm in a Shandon Cytospin 3; Shandon Scientific Ltd., Astmoor, Runcorn, UK).

Indirect immunofluorescence assays on cultured human skeletal muscle cells and isolated rabbit cardiomyocytes were performed as described above.

Confocal scanning laser microscopy (CSLM)

Immunostained adult human myocard was observed with a Bio-Rad MRC-600 confocal scanning laser microscope (CSLM; Bio-Rad Laboratories, Richmond, CA, USA), equipped with a Crypton/Argon mixed gas laser (Ion Laser Technology, Salt Lake City, UT, USA) with two separate wavelengths for the excitation of fluorescein isothiocyanate (FITC, 488 nm) and Texas Red (TR, 568 nm) and a Zeiss Axiophot microscope. To quantitate fluorescence intensities, fluorescein-labelled cryostat sections (5 μ m thick) of human

myocard were excited with a single wavelength light beam of 488 nm, using a neutral density filter no. 1.5, showing 3% transmission and a fixed pinhole position. Image processing was performed to upscale the signal and obtain optimal contrast. Photographs were taken from the monitor with a Nikon camera using a Kodak Ektachrome film with a setting of 400 ASA.

Gel electrophoresis and immunoblotting

Several procedures were applied to obtain cytoskeletal preparations from human heart muscle. Approximately 40 cryostat sections (20 μ m thick) of fresh frozen human heart tissue were collected and washed with 1 ml PBS and centrifuged for 5 min at 12,000 $\times$ g. After centrifugation the pellet was subjected to a number of different extraction steps:

1. *Triton X-100 extraction*: after the PBS wash the pellet was suspended in 1% Triton X-100, 5 mM ethylenediaminetetra acetic acid (EDTA; Merck), 0.4 mM phenylmethylsulfonyl fluoride (PMSF; Merck) in PBS, pH 7.4 [45], and extracted for 5 min on ice. After centrifugation for 5 min, 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 SDS-sample buffer [28], containing 2.3% sodium dodecylsulfate (SDS; BDH Chemicals Ltd.) and 5% β -mercaptoethanol (Bio-Rad Laboratories).

In parallel with the Triton-extraction, tissue sections were extracted with 1% Triton X-100 in PBS containing 0.025% DNase (Sigma) or 0.001% trypsin (Flow Laboratories, Irvine, UK; described by Gerdes *et al.* [16]). After treatment for 5 min with DNase or trypsin the samples were washed with PBS and dissolved in SDS-sample buffer.

2. *KI/KCl-extraction*: the pellet was homogenized and extracted for 5 min on ice in 1 ml 0.6 M KI, 1% Triton X-100, 5 mM EDTA, and 0.4 mM PMSF in PBS, pH 7.4. After centrifugation (5 min, 12,000 $\times$ g) the pellet was suspended and extracted for 5 min in 1 M KCl, 1% Triton X-100, 5 mM EDTA, 0.4 mM PMSF in PBS, pH 7.4. Finally the pellet (5 min, 12,000 $\times$ g) was washed in 1 ml PBS and dissolved in SDS-sample buffer.

3. *NaDOC/Tween40-extraction*: after the first PBS washing step, the pellet was extracted for 5 min in 0.5% NaDOC, 1% Tween40, 5 mM EDTA and 0.4 mM PMSF in PBS, pH 7.4, followed by a PBS washing step and addition of SDS-sample buffer.

For one-dimensional SDS-gel electrophoresis (SDS-PAGE) using a Mini Protean II Electrophoresis Cell (Bio-Rad Laboratories) and immunoblotting using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories) polyacrylamide slab gels containing 0.1% SDS [28] were loaded with the cytoskeletal samples, prepared as described above. After electrophoretic separation, the proteins were stained with Page Blue 83 (BDH Chemicals Ltd.) or subjected to immunoblotting.

The separated polypeptides were transferred to a nitrocellulose membrane (Filter BA 85, Schleicher and Schuell, Dassel, FRG) by blotting for 1 h at 100 V in a cold (4°C) buffer containing 25 mM Tris (Merck), 192 mM glycine (Merck), 0.02% SDS [45, 53]. No methanol was used for immunoblotting.

For detection of cardiotin with monoclonal antibody R2G the chemiluminescence method was used. After protein transfer, the nitrocellulose sheet was pre-incubated for 1.5 h in 3% bovine serum albumin (BSA; Sigma), 350 mM NaCl, and 10 mM Tris-HCl, pH 7.6. Culture supernatant of the primary monoclonal antibody R2G was 1:5 diluted and incubated overnight at room temperature in 0.3% BSA, 150 mM NaCl, 1% Triton X-100, 0.5% NaDOC, 0.1% SDS and 10 mM Tris-HCl pH 7.6, followed by washing steps (3 times 10 min) with TP-buffer: 0.5% Triton X-100 in PBS. Thereafter, the blots were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgM (DAKOpatts) for 1 h at a dilution of 1:500. The blots were washed for 2 h in TP-buffer and treated for 1 min with the chemiluminescence fluid ECL (Amersham International plc, Amersham, UK). Finally a Fuji medical X-ray film RX (Fuji Photo Film Co. Ltd., Japan) was exposed to the nitrocellulose sheets for 30 sec to 5 min.

For the detection of other muscle antigens, the nitrocellulose sheets were preincubated for 1 h at room temperature with 0.05% Tween20 (Sigma) in PBS. All antisera were diluted in 0.05% Tween20/PBS, that was also used for all washing steps. Primary antibody incubations were carried out overnight. After three washing steps (each 10 min), horseradish peroxidase-conjugated rabbit anti-mouse Ig (DAKOpatts) was applied for 1 h at room temperature in a 1:400 dilution. The blots were washed again three times for 10 min and at least one time with PBS for 5 min, followed by detection of peroxidase with 4-chloro-1-naphthol (Merck) and 0.12% hydrogen peroxide (Merck) in PBS. The blots were then rinsed with tap water for 5 min and air-dried. Immunostained blots could be reincubated one or two times with other primary antibodies [45].

Metabolic labelling and immunoprecipitation

Human satellite cells were enzymatically isolated from normal adult human skeletal muscle, grown, trypsinized and frozen as described before [56]. In our experiments, cells were quickly thawed and plated in culture dishes ($\varnothing = 60$ mm; Greiner, Frickenhausen, FRG) in a culture medium consisting of Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 20% fetal calf serum (FCS, Gibco BRL, Paisley, UK), 2% chick embryo extract (Flow Laboratories), 2 mM glutamine (Gibco BRL), 100 U.ml⁻¹ penicillin (Gibco BRL) and 1 μ g.ml⁻¹ streptomycin (Gibco BRL). When cells were grown to near confluency, they were cultured for 1 h in 1 ml/dish methionine free DMEM (Sigma) supplemented with 20% dialyzed FCS, glutamine and antibiotics as above. Subsequently 100 μ Ci [³⁵S]-methionine (Tran[³⁵S]-Label, ICN Radiochemicals, Irvine, CA, USA) was added per dish. After 16 to 20 h the dishes were washed three times with cold PBS and the cells were collected in 0.1 M Na₂HPO₄ (Merck), 0.2% BSA, 0.1% Na-azide (Merck) and 1% Triton X-100 using a rubber policeman. SDS and β -mercaptoethanol were added to 2% and 1% respectively, the samples were heated for 10 min at 90°C and centrifuged for 15 min at 12,000 $\times$ g. The supernatants were collected, 4 volumes of 6 mM EDTA, 190 mM NaCl, 2.2% Triton X-100, and 50 mM Tris-HCl, pH 7.4, and 50 μ l protein A-sepharose CL-4B (Pharmacia, Uppsala, Sweden, 1:1 in PBS) were added. This mixture was incubated for 4 h at room temperature and centrifuged for 15 min at 1,000 $\times$ g. Supernatants were collected and incubated for 16 h at 4°C with 200 μ l tissue culture supernatants of R2G or an other unrelated IgM mouse monoclonal antibody. As mouse IgM do not bind to protein A, a two step procedure was necessary, and 5 μ l rabbit anti-mouse IgM (0.5 mg.ml⁻¹, Zymed, San Fransisco, CA, USA) was added. After an incubation for 2 h at room temperature, 0.1 volume protein A-sepharose CL-4B was added and the samples were again incubated for 1 h at room temperature. The protein A-sepharose was collected by centrifugation and washed 5 times with 0.5 M NaCl, 50mM Tris-HCl pH 7.4, 1% Triton X-100, 0.1% SDS, 15 mM β -mercaptoethanol, and 5 mM EDTA. After the last washing step, 25 μ l SDS-sample buffer was added to the pellet, the samples were boiled for 10 min and electrophoresed on a 10% polyacrylamide gel containing 0.1% SDS using a Mini Protean II Electrophoresis Cell (Bio-Rad Laboratories). Gels were dried using a Bio-Rad 543 gel dryer and exposed to Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY, USA) for 6 days.

RESULTS

The newly developed monoclonal antibody R2G was characterized in immunohistochemical assays and by immunoblotting. The double-labelling immunofluorescence technique, combining R2G with several antibodies to muscle-specific constituents, was used on cryostat sections of normal tissues in order to establish the tissue distribution and subcellular localization of the protein recognized by this antibody. The newly characteri-

Figure 1: Double-label immunofluorescence microscopy of adult human heart tissue sections incubated with the monoclonal antibody to cardiotin (R2G; A, C, E, G), and with antibodies to desmin (DE-R-11 and RD301; B, D, respectively), vimentin (RV203; F) and desmoplakins 1 and 2 (Dp1&2-2.15; H). Note that smooth muscle structures of blood vessels do not stain with the cardiotin antibody (A, E) in contrast to the desmin (B) and vimentin (F) antibodies. Bar indicates 20 μm for C, D; 40 μm for A, B, G, H; 80 μm for E, F.

zed protein is designated cardiotin, because it is mainly restricted to cardiac striated muscle [46, 47].

Distribution of cardiotin in human tissues

The monoclonal antibody R2G, from here on designated as anti-cardiotin, reacts strongly with adult human heart muscle cells and to a lesser extent with human skeletal muscle cells. A fibrillar reaction pattern was observed between the myofibrils of the heart cells (Fig. 1A, C). The typical cardiotin staining pattern was observed in different regions of the adult human heart i.e., the right and left ventricle and the right and left atrium as well as in the apex of the heart.

In striated muscle cells of different tissues, such as tongue and skeletal muscle, a more diffuse staining pattern was obtained (Fig. 2A), and the fibrillar pattern, as seen in the myocard, was not as evident. Neither in the heart nor in other striated muscle cells, cross-striation of cardiotin was found. No cardiotin was found in smooth muscle cells surrounding blood vessels (compare Fig. 1A and 1E with 1B and 1F) or in smooth muscle layers of the small intestine, colon, stomach, and esophagus (compare Fig. 2C with 2D). Also in other tissues of non-muscle origin, such as epithelia (see: Materials and Methods) and stroma, no cardiotin was detected using the monoclonal cardiotin antibody. In double-label immunofluorescence assays of adult human myocard sections, co-distribution of cardiotin and titin in the same cells was evident (Fig. 3A, B), while these cardiomyocytes also contained desmin but no vimentin.

Species cross-reactivity of the anti-cardiotin antibody

The monoclonal anti-cardiotin antibody was tested on frozen sections of heart, tongue, esophagus (in some species containing striated muscle cells), skeletal muscle, and smooth muscle layers of the small intestine dissected from different animal species. The sections were pretreated routinely with 0.5% Triton X-100 in PBS. In comparison with cardiotin localization in the human myocard, a similar fibrillar reaction pattern between the myofibrils was seen in heart tissue of Rhesus monkey (Fig. 4A), cow, goat (Fig. 4C), dog, cat, rabbit (Fig. 4E, F), mouse (Fig. 4D), chicken, and *Xenopus laevis*. In rat heart tissue no cardiotin could be detected.

In cytospin preparations of isolated adult rabbit heart myocytes a strong cardiotin reaction pattern was found between the myofibrils with anti-cardiotin antibody (Fig. 4G). Similar to the situation in man, cardiotin filaments are perpendicularly localized to the characteristic cross-striated pattern of desmin (Fig. 4H).

In striated muscle cells from tongue, esophagus and skeletal muscle of the tested animals, with exception of the rat, a relatively weak and diffuse cardiotin staining pattern was detected. In rat striated muscle cells no cardiotin could be detected. In all animal tissues examined smooth muscle cells, stroma and epithelia were negative for cardiotin.

Age-related expression of cardiotin

From our immunohistochemical assays with different species and different tissues from mammals of different age, it became apparent that expression of cardiotin in the myocard is age-dependent.

In a 16-week-old human embryonic heart tissue no cardiotin was found (Fig. 5A), although vimentin was found to stain connective tissue and no cardiomyofibrils (Fig. 5B), while titin and desmin both showed a striated staining pattern (Fig. 5C, D). As mentioned above adult human heart tissue is strongly positive for cardiotin.

Figure 2: Double-label immunofluorescence microscopy of adult human skeletal muscle (A, B) and adult smooth muscle of human esophagus (C, D), incubated with the monoclonal antibody to cardiotin (A, C), a monoclonal desmin antibody (DE-R-11; B) and a polyclonal antibody to desmin (pDes; D). Bar indicates 25 μm for A, B; 50 μm for C, D.

Figure 3: Adult human myocard sections after extraction with 0.5% Triton X-100 (A, B), 0.5% Triton X-100 and NaDOC/Tween40 (C) or 0.5% Triton X-100 and KI/KCl (D), incubated with the monoclonal antibody to cardiotin (R2G: A, C, D) and a polyclonal titin antiserum (pTitin; B, double-staining of A). Bar indicates 80 μm for A-C; 40 μm for D.

Figure 4: Immunofluorescence micrographs of heart sections of Rhesus monkey (A, B), goat (C), mouse (D), and rabbit (E, F) stained with the monoclonal antibody to cardiotin (A, C-F). In Fig. 4A, B a double-label immunofluorescence staining of adult Rhesus monkey heart (20 years old), stained with the monoclonal cardiotin antibody (R2G; A) and a monoclonal antibody to desmin (DE-R-11; B) respectively. In Fig. 4G, H isolated cardiomyocytes of rabbit stained with R2G (G) and pDes (H). Bar indicates 12.5 μ m for G, H; 20 μ m for F; 40 μ m for A, B; 80 μ m for C, D, E.

Figure 5: Immunofluorescence micrographs of a 16 weeks old human embryonic myocardium (A-D) and young Rhesus monkey heart (E, F) stained for cardiotin (R2G; A, E), vimentin (RV203; B), titin (9D10; C), and desmin (pDes; D and DE-R-11; F). Note the absence of cardiotin in the human embryonic myocardium in contrast to the young Rhesus monkey myocardium in which the presence of cardiotin can be detected by R2G. Bar indicates 30 μ m for A-D; 40 μ m for E, F.

In rabbit embryos of different stages of development (8, 10, 13, 18, 23, and 28 days post coitum (d.p.c.)) no cardiotin was detected, neither in the developing heart region, nor in the myotomes. We have described before [55] that during cardiomyogenesis of the rabbit heart titin is expressed as early as the 0 to 1 somite pair embryos, and is one of the earliest markers to detect the heart-anlagen. Cardiomyofibrils with a clearcross-striated pattern of titin or desmin were observed in rabbit embryos of 5 to 8 somites (8.0 - 9.0 d.p.c.) and >30 somites (13 d.p.c.), respectively.

In frozen heart sections of 5 months and 13 months old Rhesus monkeys, incubated in

Figure 6: Immunofluorescence study of cultured human skeletal muscle cells (A - H). Fig. 6A-F show undifferentiated human skeletal muscle cells *in vitro* stained with anti-cardiotin (A, C, E) and double-stained with polyclonal antisera directed to desmin (pDes; B, D) and titin (pTitin; F). Double-label immunofluorescence (G, H) of differentiated human skeletal muscle cells *in vitro* stained with the monoclonal antibody to cardiotin (G) and the polyclonal titin antiserum (H). Bar indicates 25 μm for A, B, E-H; 12.5 μm for C, D.

double immunofluorescence assays, a relatively weak, occasionally diffuse staining pattern was detected for cardiotin (Fig. 5E). In these tissue sections not all the cardiomyofibrils were cardiotin positive. With the desmin monoclonal antibody (DE-R-11) a clearly cross-striated staining pattern was detected in both specimens (Fig. 5F), throughout the complete myocard. Also the intercalated discs were stained for desmin in these heart sections of the young monkeys. In the adult, 20 years old, monkey heart (Fig. 4A, B) a normal staining pattern, comparable to that seen in the adult human heart, was detected for cardiotin and desmin.

Cardiotin in cultured human skeletal muscle cells

In 25% of cultured, postmitotic mononuclear human skeletal muscle myoblasts, a typical perinuclear punctate staining pattern was found for cardiotin in the cytoplasm (Fig. 6A, C), while in all of these polygonal muscle cells a filamentous staining pattern was found for desmin (Fig. 6B, D). In the double-label immunofluorescence assays of these undifferentiated human skeletal muscle cells no co-localization was found when the punctate cardiotin pattern was compared to the characteristic pattern of spots obtained with a polyclonal titin antiserum (Fig. 6E, F). Note that not all the cardiotin positive cells are positive for titin.

In the *in vitro* differentiated multinuclear myotubes no intracellular staining was found for cardiotin (Fig. 6G), while a filamentous to stress fiber-like staining pattern was detected for titin (Fig. 6H).

Subcellular localization of cardiotin in the myocard

Immunofluorescence assays of adult human cardiomyocytes show cardiotin filaments to be located intracellularly between the cardiomyofibrils, perpendicular to the sarcomere striated pattern as detected by desmin (Fig. 1C, D) and titin (Fig. 3A, B). The individual filament structures running parallel to each other span many myofibrillar sarcomeres. As determined by means of CSLM the length of the cardiotin filament structures ranged from 12 to 80 μm . The distance between cardiotin filaments seems to be constant in most cases, showing an average distance of 2.3 μm as determined after 100 measurements

Figure 7: Histogram showing the distribution of the distance between cardiotin filaments as measured by CSLM. One-hundred estimates of the distance between parallel running cardiotin filaments were performed.

(Fig. 7). Combining the cardiotin antibody with an antiserum to the basal membrane constituent collagen type IV, showed that cardiotin filaments were always located inside the muscle cells (results not shown). In double-label immunofluorescence assays cardiotin could not be detected in the intercalated discs (Fig. 1G, H) which are strongly positive with antisera to desmin (Fig. 1D) and

Figure 8: Immunoblottings of cardiotin in extracted human heart tissue sections. lane A) Page Blue stained gel of normal human heart extracted with 1% Triton X-100 in PBS containing 0.001% trypsin. lanes B and C) Immunoblots of normal human heart incubated with the monoclonal antibody to cardiotin (lane B) and myosin (lane C). lane D) Lane B subsequently incubated with a myosin antibody (MF20). lane E) Desmin as detected with RD301. c: cardiotin (± 300 kDa); m: myosin (200 kDa); d: desmin (53 kDa).

Cardiotin is a high molecular mass protein

For the determination of the molecular mass of the cardiotin protein subunit, Triton X-100 extracted 20 μ m thick cryostat sections of fresh frozen human heart were analyzed after SDS-PAGE and immunoblotting. A weak immunoreaction with a high molecular mass (> 300 kDa) protein band, migrating just below at the interface between the stacking and the running gel was found with anti-cardiotin in blots of 12% acrylamide gels. After extraction of 20 μ m myocard sections with KI/KCl or NaDOC/Tween40 in the presence of 1% Triton X-100 a similar result was obtained (result not shown). A slightly stronger staining of this high molecular mass protein could be detected when human heart samples were treated with 0.001% trypsin (Fig. 8, lane B) or 0.025% DNase (result not shown) in the presence of 1% Triton X-100. To determine the molecular mass of cardiotin more precisely, the same blot was reincubated with a monoclonal antibody to sarcomeric muscle myosin (MF20) detected at the 200 kDa molecular mass level (Fig. 8, lane C). After the subsequent incubation of the blot with R2G and anti-myosin, it is clearly seen that the 200 kDa myosin migrates further into the gel than the cardiotin band (Fig. 8, lane D). An exact molecular mass for cardiotin can not be given sofar. As a control, desmin was detected in these samples, showing a 53 kDa protein without significant breakdown products (Fig. 8, lane E). Immunoprecipitation studies with radio-labelled extracts from cultured human skeletal muscle cells also revealed a protein band, migrating above the 200 kDa marker protein in a 10% polyacryl-amide gel (Fig. 9).

desmoplakins (Fig. 1H). In fact it seemed that the cardiotin fibrils did not cross this boundary. In transverse sections of the myocard, cardiotin was also found to be located between the emyofibrils (Fig. 3C, D).

Cardiotin is a highly insoluble structural component of the myocard

Extraction of human heart cryostat sections with 1% Triton X-100 apparently had no significant effect on the cardiotin structure as concluded from the immunofluorescence staining pattern (Fig. 3A). A KCl/KI extraction step or treatment of the sections with NaDOC/Tween40 solutions had also no dramatic effects on the staining results of the cardiotin antibody (Figs 3C, D). A very weak reaction was found after methanol/acetone fixation of the tissue, indicating that the anti-cardiotin epitope is sensitive to fixation in organic solvents.

Figure 9: Autoradiographs of 10% SDS-polyacrylamide gels containing: lane A) Radiolabelled molecular mass markers; lane B) Triton X-100 extract of cultured human skeletal muscle cells, immuno-precipitated with the monoclonal antibody to cardiotin; arrowhead indicates the specifically precipitated cardiotin protein band at a molecular mass level of approximately 300 kDa; lane C) Human skeletal muscle cell extract as in lane B, immuno-precipitated with an unrelated monoclonal antibody of the IgM-class, used as a control.

DISCUSSION

In the present study we have determined the tissue-distribution and subcellular localization of the newly identified component cardiotin [46, 47] that exhibits a typical distribution pattern in the myocard.

Tissue distribution of cardiotin

In different regions of normal adult human heart cardiotin is abundantly expressed only in the striated muscle cells, and not in connective tissue, blood vessels or nerve tissue. Cardiotin is localized in the same cells that also contain titin.

In cross-striated muscle cells of human tongue and skeletal muscle the cardiotin antibody showed a more or less diffuse staining reaction. In contrast to what was detected in the myocard no clear organization of cardiotin could be found in these muscle cells. When examining an extended panel of different tissues, no cardiotin staining was found in epithelia (squamous, glandular or transitional), nor in connective tissue, smooth muscle cells, brain tissue or nerve cells.

Within the different animal species that

we have examined, the cardiotin tissue distribution, showed a similar pattern as described for human tissues. The fact that this holds true for animals as distant as amphibia and birds indicates the importance of cardiotin in the proper functioning of striated muscle cells, in particular of the heart. The fact that rat myocard is negative, is probably due to a difference in the structure of the cardiotin-epitope recognized by R2G, or to masking of this region in the protein

In vitro differentiating human skeletal muscle cells revealed the presence of cardiotin in polygonal undifferentiated myoblasts. During myofibrillogenesis *in vitro* of the human skeletal muscle cells the cardiotin spots disappear very early in the differentiating process, in contrast to titin spots [56].

During embryonic development of the heart in man and rabbit, no significant expression levels of cardiotin could be detected, in contrast to other muscle-specific proteins such as titin, myosin and desmin. These are expressed very early in the developing heart of man and rabbit [55, 64]. Rhesus monkey myocard tissue obtained five months after birth did already show a fibrillar cardiotin pattern which was less abundant than the pattern seen in adult Rhesus monkey myocard. This age related expression pattern of cardiotin parallels the deposition of the sarcoplasmic reticulum (SR) around the myofibrils [7, 8]. The findings suggest a possible relation between cardiotin and these SR structures, a correlation that is also supported by the subcellular localization of the fibrillar cardiotin structures (see below).

In conclusion, cardiotin seems not to be of significant importance for the early development of the heart, but it plays a role in a proper functioning of the adult heart.

Intracellular localization of cardiotin

In myocard tissue of different species, a similar intracellular cardiotin distribution pattern became apparent, i.e., longitudinally oriented filaments, running parallel at a regular distance spanning several sarcomeres, and always within the basement membrane surrounding the cell. The average width of normal adult human cardiomyofibrils is approximately 2 μm . The average distance between the cardiotin filaments was estimated to be 2.3 μm . This strongly suggests that cardiotin in human heart is situated between these myofibrils. This was also evident from the transverse sections, where cardiotin was also seen to be located between individual fibrils. Double-label immunofluorescence studies on frozen sections of myocard with a basal membrane antibody supported this idea, and also showed that cardiotin is located intracellular. No obvious connection of cardiotin molecules with the intercalated disc seems to exist. This localization of cardiotin bears some resemblance to the distribution pattern to be expected for the SR [7, 8]. Immunoelectron microscopy studies under progress will have to reveal its exact distribution and possible association with other structures.

In striated muscle cells of the tongue, esophagus and skeletal muscle tissue, no precise localization of the cardiotin structure could be determined on basis of light microscopy. The difference in the localization of cardiotin in cardiomyocytes as compared to skeletal muscle may be of functional importance.

The typical cardiotin spots in cultured human skeletal muscle cells did not show co-localization with the titin spots, which indicates that these cells are still polygonal but have already initiated differentiation [45, 52, 56]. In fact, the distribution pattern of cardiotin observed in these cultured postmitotic skeletal muscle cells resembles that of the endoplasmic reticulum. Therefore it will be interesting to see whether or not the diffuse staining pattern in skeletal muscle cells *in vivo* reflects an association of cardiotin with the SR. When compared to the staining of the SR and transverse tubules in skeletal muscle cells as described by Flucher *et al.* [7, 8], the cardiotin pattern in skeletal muscle seemed, however, different in our studies.

Structure of cardiotin

Like titin and nebulin [33-35, 50, 54, 60] cardiotin is a high molecular mass protein restricted to striated muscle. Cardiotin subunits have a molecular mass over 300 kDa, but migrate below titin in polyacrylamide SDS-gels. The length of individual titin molecules, with an approximate molecular mass of 3.000 kDa, has been described to vary between 1 μm and 2 μm [54]. The cardiotin filaments showed a length variation of 12 μm to 80 μm as estimated by CSLM in frozen sections. This can only mean that these structures consist of cardiotin polymers. Unlike titin antibodies, the cardiotin antibody R2G shows a rather uniform staining of the filaments, no real periodicity was observed. This may mean that the epitope recognized by R2G occurs as a repeated motif within the subunit molecule. Repeated motifs have been shown to occur in other high molecular mass proteins such as titin [27, 54] and nebulin [26, 54, 66]. Alternatively a staggered assembly of overlapping cardiotin molecules may be responsible for the formation of the long cardiotin filaments, in analogy to the structure of intermediate filaments.

Future studies at the molecular level will have to reveal the basic structure of the cardiotin molecule, which will then allow to draw up a more precise model for cardiotin assembly.

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CHAPTER 5

TISSUE-SPECIFIC EXPRESSION OF A VIMENTIN-DESMIN HYBRID GENE IN TRANSGENIC MICE

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ABSTRACT

We have introduced a hybrid gene, pVVim2, composed of the 5' region of the hamster vimentin gene encoding the head and rod domain of vimentin and the 3' region of the hamster desmin gene encoding the tail domain of desmin, into the germ line of mice by pronuclear injection. RNA and protein analysis of mice transgenic for this construct showed that the pVVim2 gene was expressed at high levels in a developmental and tissue-specific manner. This indicates that the vimentin-derived segment of the fusion gene contains all the regulatory elements required for vimentin-specific expression. Immunohistochemical staining of fibroblast cultures derived from the transgenic mice with antibodies specific for vimentin and desmin demonstrated that the pVVim2 protein is assembled into filaments that colocalize with the endogenous vimentin filaments. The expression of pVVim2 protein in mesenchymal cells does not interfere with the function of vimentin in these cells.

INTRODUCTION

The intermediate filaments (IFs) have been characterized as a unique set of cytoskeletal structures, composed of cell type-specific proteins. These proteins can be divided into five distinct classes, which are expressed in a developmentally regulated and tissue-specific fashion [7, 16]. For example, desmin is only expressed in muscle cells, while vimentin is almost exclusively found in cells of mesenchymal origin [13]. In addition, vimentin is found in many cells cultured *in vitro* [6, 31, 33]. The intermediate filament proteins are strongly related to the nuclear lamins and have been assigned to the same multigene family [5, 14]. The IF subunits have a similar structural organization: a central conserved α -helical domain (rod) of ~320 amino acid residues is flanked by a non-helical amino-terminal head domain and a carboxy-terminal tail domain of variable length [8, 29]. *In vitro*, the head and rod domains play a pivotal role in the polymerization of IF subunits [12, 30].

The close relationship between the IFs is also reflected in their gene structure. The exon-intron organization of vimentin, desmin and the glial fibrillary acidic protein is similar and coding sequences show up to 65% homology (in case of vimentin and desmin) [18, 20, 27]. For most IF genes little is known about the regulatory sequences controlling their developmental and tissue-specific expression. For hamster desmin we have shown that the region between -89 bp and +25 bp relative to the cap site is sufficient for cell type-specific transient expression. The flanking 5' region of the vimentin gene contains several regulatory elements. These elements are instrumental both in the downregulation of vimentin gene expression during myogenesis [17] and in the transcriptional modulation of vimentin gene expression during myogenesis [17, 26].

Determination of the cell type-specific function of vimentin and the characterization of the regulatory pathways controlling vimentin gene expression is partly hampered by the fact that expression of vimentin is induced in most cells by *in vitro* culture. Therefore, we have introduced a vimentin-desmin hybrid gene construct into the germ line of mice to study the tissue-specific regulation of vimentin gene expression *in vivo* and to obtain insight in tissue-specific IF functions. In this construct the last three exons of the hamster vimentin gene covering the complete tail domain were replaced by the last three exons of the hamster desmin gene. This allows recognition of the gene product in the presence of endogenous vimentin, while retaining the structural features characteristic for intermediate filaments. The expression of this fusion gene was analyzed in transgenic mice and in cell lines derived from these mice.

MATERIALS AND METHODS

Plasmid construction

Isolation and characterization of the hamster vimentin and desmin genes have been described previously [18-21]. Intervening sequences map at similar positions in the gene. The last three exons of the vimentin gene were replaced by the corresponding exons of the desmin gene. The 9.5 kbp BamHI-BglII fragment harboring the first six exons of vimentin was subcloned into the BamHI site of the pUC19. A 3.4 kbp EcoRI-StuI fragment containing the last three exons of desmin was fused to the 3' end of the vimentin fragment via the EcoRI site in the polylinker region. The complete pVVim2 hybrid gene is present on a 13 kbp BamHI fragment. As hybridization probe specific for hamster vimentin M13 phage E49 was used, which contained a Sau3A fragment covering the region between 150 nucleotides upstream to 370 nucleotides downstream from the cap site [18]. For hamster desmin the M13 phages X54 (a Sau3A insert, containing the last 25 nucleotides of exon 8, intron 8 and the first 350 nucleotides of exon 9) and AA85 (a PstI-TaqI insert corresponding to nucleotides 165 to 357 downstream from the stop codon [20]) were used.

Cell culture

Cell cultures from ear fibroblasts were established as described [2].

Transgenic mice

Fertilized mouse eggs were recovered in cumulus from oviducts of super-ovulated (CBA x C57Bl/LiA) F1 females that had mated with F1 males several hours earlier. Approximately 200 copies of the pVVim2 hybrid gene construct (without plasmid sequences) were microinjected in the most accessible pronucleus. Microinjected eggs were implanted into oviducts of 1-day pseudopregnant MA or F1 foster mothers and carried to term. Total genomic DNA was prepared from tail biopsies 3 to 4 weeks after birth. For Southern blot analysis 8 μ g of total genomic DNA was digested with BamHI or StuI, run on a 0.6% agarose gel and transferred to nitrocellulose. The filter was hybridized to 32 P-labelled probes as described [4].

Northern blot analysis

Fifteen μ g of total cellular RNA, prepared by the LiCl-urea method [1] was separated on a 1% agarose gel in formaldehyde and transferred to nitrocellulose.

Immunohistochemical analysis

The intermediate filament protein expression of tissues and cell lines was assayed by indirect immunofluorescence and Western blot analyses. The antisera used for this purpose include:

1. An affinity-purified polyclonal antibody directed against human skin keratins (pKer). This antibody reacts with virtually all epithelial tissues but not with non-epithelial cells [24].
2. An affinity-purified polyclonal antibody to bovine lens vimentin (pVim) [24].

3. A polyclonal rabbit antibody to chicken gizzard desmin (pDes) [25].
4. A mouse monoclonal antibody RV202 to vimentin, directed against bovine lens vimentin [3].
5. A mouse monoclonal antibody RD301 to desmin, raised against chicken gizzard desmin [3].

As second antibodies for indirect immunofluorescence assays either fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG or FITC-conjugated rabbit anti-mouse IgG were used at a dilution of 1:25 (Nordic, Tilburg, The Netherlands). For double-label immunofluorescence FITC-conjugated goat anti-rabbit IgG was combined with Texas Red (TR)-conjugated sheep (Fab')₂ anti-mouse Ig (New England Nuclear, Boston, USA). In control experiments phosphate-buffered saline (PBS: 137 mM sodium chloride (Merck, Darmstadt, FRG), 13 mM disodium hydrogen phosphate dihydrate (Na₂HPO₄·2H₂O), 3 mM potassium dihydrogen phosphate (KH₂PO₄; Merck), pH 7.4) was substituted for the primary antibody. Cells on coverslips and 5 µm thick frozen sections of mouse tissues were fixed in methanol (Merck) for 5 sec (-20°C) and acetone (Merck; 3 times 5 sec), air-dried and incubated with the primary antibody for 30 to 45 min at room temperature. Further processing for indirect immunofluorescence was performed as described [3].

Gel electrophoresis and immunoblotting assays

Cytoskeletal preparations of eye lens were made as follows: lenses were suspended in PBS containing 1% Triton X-100 (BDH Chemicals Ltd., Poole, UK), 0.4 mM phenylmethylsulfonyl fluoride (PMSF; Merck) and 5 mM EDTA (Merck) for 10 min at 4 °C. After centrifugation (3,000xg for 10 min) and washing (PBS) the pellet was dissolved by boiling during 5 min in SDS-sample buffer. For the crude desmin preparation from mouse skeletal muscle, frozen 5 µm thick sections were first extracted with 1.5 M KCl (Merck), 0.5 % Triton X-100, 5 mM EDTA, 0.4 mM PMSF in PBS, and processed as described above. One- and two-dimensional SDS-gel electrophoresis and immunoblotting was performed as described [3].

RESULTS

The pVVim2 construct

For the construction of the vimentin-desmin hybrid gene, further referred to as pVVim2 gene, the hamster vimentin and hamster desmin genes served as starting material. Figure 1 shows a physical map of both genes. Introns in both genes map at identical positions [18, 20]. In the pVVim2 construct a 9.2 kbp BamHI-BglII fragment comprising the promoter region and the first six exons encoding head and rod domain of the vimentin gene were fused via the EcoRI site in the pUC19 polyliner to a 3.4 EcoRI-StuI fragment harboring the last three exons of the desmin gene encoding the tail domain. In this construct 3.1 kbp of 5' flanking DNA is present. No other constructs were examined for tissue-specific expression in transgenic mice in the study. The pVVim2 gene construct is depicted in figure 1A, and the composition of the expected hybrid protein in figure 1B. Amino acids 1-408 comprise the head and rod domain of vimentin and amino acids 408-463 the tail domain of desmin. Cotransfection of the pVVim2 plasmid with the pSV2-neo plasmid into tissue culture cells (HeLa and hamster lens) results in stable transformants expressing the pVVim2 gene, as assayed with a polyclonal desmin antiserum [32]. The data show that the hybrid protein encoded by the pVVim2 construct is able to assemble into a cytoskeletal network with normal IF appearance.

Figure 1: A) Physical map of the hamster vimentin gene, the hamster desmin gene and the hybrid gene pVVim2. Bars represent exon sequences; BamHI, BglII, EcoRI, and StuI restriction sites are indicated as B, Bg, E and S, respectively; PL represents the polylinker of the pUC19 vector. B) Schematic representation of the hybrid protein encoded by the pVVim2 gene.

Transgenic mice

For the generation of transgenic mice the pVVim2 hybrid gene was excised from the vector and injected into the most accessible pronucleus of fertilized mouse eggs. Three transgenic mice were obtained (Nos. 32, 34 and 44). Southern blot analysis of tail DNA showed that the three transgenic founders had incorporated multiple copies of the gene in a head-to-tail arrangement (Figure 2). None of the transgenic mice showed any abnormalities. Two founders (Nos. 32 and 44) transmitted the pVVim2 gene to their offspring. Southern blot analysis indicated that founder No. 44 was mosaic for the pVVim2 gene, as its offspring showed a much stronger hybridization signal. This was also confirmed by immunofluorescence studies on tissue sections (see below).

Tissue-specific expression

The expression of the pVVim2 protein was analyzed by the indirect immunofluorescence

Figure 2: Southern blot analysis of tail DNA from transgenic mice. 8 μ g of DNA was digested with *Stu*I, run on 0.6% agarose gel, transferred to nitrocellulose and hybridized with the desmin-specific probe AA85; *Stu*I cleaves once in the pVVim2 fragment and in a head-to-tail tandem array of pVVim2 copies it generates a single hybridizing fragment of unit size; in all transgenic mouse lines multiple copies of pVVim2 were integrated. Lane 1) DNA from a control mouse; lane 2) DNA from founder No. 32; lane 3) DNA from offspring of founder No. 32; lane 4) DNA from founder No. 34; lane 5) DNA from founder No. 44; lanes 6) and 7) DNA from offspring of founder No. 44.

(pKer; data not shown). In frozen sections from transgenic spleen nearly all cells stain with pDes, whereas in spleen from control mice only smooth muscle tissue of blood vessels are positive (data not shown). In testis of transgenic mice Leydig cells and Sertoli cells express the hybrid protein (Fig. 3h). In liver of pVVim2 transgenic mice, Kupffer cells which express vimentin stain strongly with the pDes serum (Fig. 3k). A comparison of liver sections from founder No. 44 with liver sections from its positive offspring confirmed the mosaic character of transgene integration in founder No. 44; only Kupffer cells in distinct liver segments react with the pDes antiserum in the founder (Fig. 3m), whereas in positive offspring all Kupffer cells stain with this antibody.

Identification of the pVVim2 mRNA and protein *in vitro*

Ear-shell fibroblasts from transgenic founder No. 32 were immortalized by SV40, and the cells were cloned and used for RNA and protein analysis. It appears that the hybrid pVVim2 protein is present in a filamentous network with normal IF appearance (Figure 4). Double-label immunofluorescence studies of the same cells with desmin and vimentin antibodies show that the hybrid protein is colocalized with the endogenous vimentin filaments (Fig. 4c, d).

RNA was isolated from these cell lines and analyzed by Northern blot (Figure 5). Clearly, only in fibroblastic cell lines derived from a pVVim2 transgenic mouse was an mRNA of the appropriate size (2.2 kbp) observed with the desmin probe X54, whereas fibroblasts from control mice were nonreactive. Hybridization with the vimentin-specific probe E49

technique on tissue sections using polyclonal rabbit antisera directed against desmin (pDes), vimentin (pVim) and cytokeratin (pKer) [24, 25]. The immunohistochemical analyses were performed on blood cells, sections from tailskin, earshell, liver, spleen and testis. For blood cell analyses, blood was collected from the tail vein and smeared onto cover slips. Blood cells from transgenic mice displayed intensive staining after incubation with the pDes antiserum, whereas cells from control mice were non-reactive (Fig. 3a-c'). Staining of macrophages was most pronounced. The staining patterns often show a filamentous organization. This suggests that the pVVim2 molecules are able to assemble into intermediate filaments. Sections of tailskin and earshell from transgenic line 44 were used to analyze the tissue-specific expression of the pVVim2 gene. A strong staining with the pDes antiserum was observed in all tissues that normally express vimentin e.g., fibroblasts and cartilage (Fig. 3d-f). No reaction was seen, however, in epithelial tissues (epidermis and hair-root sheath) that stained with the polyclonal keratin antiserum

Figure 3: Indirect immunofluorescence studies. Immunofluorescence was performed on blood smears (a-c'), frozen sections of tail skin (d-f), testis (g-i) and liver (j-m) from control mice (a,d,g,j), positive offspring of founder No. 44 (b, c, e, f, h, i, k, l) and of founder No. 44 showing mosaic expression (m). Sections depicted in panels a-h, j, k and m are reacted with the pDes antiserum; i and l are stained with the pVim antiserum. Note that incubation of the smears and tissues from control mice with a polyclonal desmin antiserum does not show any staining of blood cells (a) or epidermis (d). Staining is exclusively seen in cells known to express desmin, i.e., smooth muscle cells of blood vessel walls (j) or myoid cells surrounding the seminiferous epithelium of the testis (g). In mice harboring the pVVim2 transgene, cells normally expressing only vimentin, i.e., macrophages (b, c), fibroblasts (e, f), Leydig cells and Sertoli cells in testis (h), and Kupffer cells in liver (k, m), now also express the pVVim2 protein, as visualized with the pDes antiserum. For comparison the frozen sections were also incubated with pVim (i,l). Mosaic expression can be seen in the liver section depicted in m.

Figure 4: Double-label immunofluorescence staining of transformed fibroblasts from transgenic mouse No. 32. Colocalization of pVim2 protein and vimentin filaments is shown by staining with the polyclonal vimentin antibody (pVim; c) and the monoclonal desmin antibody (RD301; d). Fibroblasts from control mice react with pVim (a) and not with RD301 (b).

showed the presence of the 2.0 kbp long mouse vimentin mRNA in cell lines from both control and transgenic mice.

The hybrid gene is expressed at a level similar to that of the endogenous vimentin. Comparison of mRNA levels in fibroblasts from transgenic and control mice (Figure 5, compare lanes 1 and 2 to lane 3) clearly shows that the expression of the endogenous vimentin mRNA is not influenced by pVim2 expression.

Identification of the pVim2 protein

The immunohistochemical data were further substantiated by biochemical identification of the pVim2 protein. Protein analyses by Western blotting were performed on the SV40-transformed fibroblastic pVim2 positive cell clones which were also used for RNA analyses. In addition the *in vivo* synthesized pVim2 protein was characterized. We chose lens cell tissue which normally expresses vimentin [23].

Western blots of SDS-polyacrylamide gels were incubated with a monoclonal antibody to desmin (RD301), and with a monoclonal antibody specific for vimentin (RV202). The epitope recognized by RD301 is located in the tail domain of desmin encoded by the last three exons of the hamster desmin gene [32]. Lysates from BHK21/C13 cells and a crude desmin preparation from mouse muscle were used as positive controls. SV40-transfor-

Figure 5: Northern blot analysis of RNA from fibroblastic cell lines derived from pVVim2 transgenic mice. 15 μ g total RNA was electrophoresed on a 1% agarose gel, transferred to nitrocellulose and hybridized with desmin-specific probe X54 (panel A), and with the vimentin-specific probe E49 (panel B). Lanes 1) and 2) RNA isolated from two independent pDes positive cell lines derived from founder No. 32; lane 3) RNA isolated from a pDes negative control cell line.

med fibroblasts and lenses from control mice were used as negative controls. The results (Figure 6) clearly show the presence of the pVVim2 protein with an apparent molecular mass of 57 kDa in the transgenic fibroblastic cell lines and lens cells. In skeletal muscle tissue no pVVim2 or vimentin protein was detected (Fig. 6, lane 3). Since we used different antibodies to detect the hybrid protein it seems unlikely that this negative reaction is caused by masking of epitopes due to conformational change during myogenesis. This was confirmed by immunohistochemical analyses of muscle tissue (data not shown). Both striated and smooth muscle cells from different organs were stained with monoclonal and polyclonal antibodies against vimentin and desmin. In transgenic mice the desmin staining pattern was similar to that in control mice. No staining with the vimentin antibodies was observed in striated muscle cells indicating that both pVVim2 and vimentin are not expressed in these cells. Smooth muscle cells which do not express vimentin in control mice also do not express pVVim2 or vimentin in transgenic mice.

The pVVim2 expression level

In order to determine the expression level of pVVim2, cytoskeletal fractions of eye lenses from control and transgenic mice (founders Nos. 32 and 44) were analyzed by one-dimensional SDS-gel electro-phoresis (Fig. 7A). In lenses of offspring from founder No. 44

Figure 6: Biochemical characterization of intermediate filament proteins from tissues of transgenic and control mice. Western blots prepared from tissue proteins were incubated with the monoclonal desmin antibody RD301 (panel A), and subsequently with the monoclonal vimentin antibody RV202 (panel B). Lane 1) transformed fibroblasts from control mouse; lane 2) transformed fibroblasts from transgenic mouse No. 32; lane 3) muscle, only positive for desmin; lane 4) BHK cells, positive control for desmin and vimentin; lane 5) eye lens from transgenic mouse No. 32; lane 6) lens from control mouse; lane 7) BHK cells; additional protein bands below desmin represent characteristic IF breakdown products.

Figure 7: Biochemical characterization and analysis of expression levels of IF proteins from eye lenses of transgenic and control mice. A) Coomassie Brilliant Blue (CBB) staining of one-dimensional SDS-gels of cytoskeletal preparations from control and transgenic mice. Lane 1) lens extract from control mouse; lane 2) lens extract from transgenic mouse, derived from founder No. 44; lane 3) lens extract from transgenic mouse derived from founder No. 32. Arrow indicates pVVim2 protein and vimentin position. C: crystallin polypeptide chains serve as standards for the amount of protein applied onto the gel. B) Total lens extract of control (1-3) and transgenic mice (4-6), analyzed by two-dimensional gel electrophoresis and stained with CBB (1, 4) or immunoblotted (2, 3, 5, 6) and stained with the monoclonal desmin antibody RD301 (2, 5), and subsequently with the monoclonal vimentin antibody RV202 (3, 6). Both mice were derived from founder No. 44. Arrows indicate IF protein position. Additional spots on the blot represent characteristic IF breakdown products.

high levels of IF expression were detected clearly exceeding vimentin expression in lenses from control mice. Lenses of offspring from founder No. 32 also showed elevated, although much less, IF expression levels. 2D-gel electrophoresis analyses of total lens extracts from control and transgenic offspring from founder No. 44 confirmed these findings (Fig. 7B). Immunoblotting of 2D-gels positively identified the hybrid protein and vimentin which were not separated on these gels. Identical results were obtained with cytoskeletal preparations (data not shown).

DISCUSSION

In this paper we describe the construction and transgenic expression of a hybrid gene between the genes of the cytoskeletal proteins vimentin and desmin. A DNA fragment containing 3.1 kbp of 5' flanking DNA and the 5' part of the vimentin structural gene up to the 6th exon, encoding the head and rod of the vimentin, was fused to the three last exons of the desmin gene encoding the tail portion of desmin. This chimeric gene, pVVim2, was introduced into the germ line of mice and its expression was analyzed at the mRNA and protein level using specific DNA probes and immunoreagents. The data show that a properly sized mRNA is transcribed from the pVVim2 gene. The pVVim2 mRNA is translated into a protein of the expected molecular mass.

Immunohistochemical analyses of tissue sections indicate that the expression pattern of the transgenic pVVim2 gene is indistinguishable from that of the endogenous vimentin. The expression levels differ in the two transgenic mouse strains investigated. Offspring mice from founder No. 32 expressed the hybrid gene at levels similar to the endogenous vimentin gene, whereas offspring from founder No. 44 expressed pVVim2 to a signifi-

cantly higher level. RNA analyses show that endogenous vimentin expression is not measurably influenced by transgenic pVVim2 expression.

All the regulatory sequences required for the tissue-specific expression of vimentin are included in the 9.2 kbp BamHI-BglII fragment, starting 3.1 kbp upstream from the transcriptional initiation site. The immunofluorescent staining pattern with the polyclonal desmin antiserum (pDes), especially of transformed fibroblasts (Fig. 4) demonstrates that the pVVim2 protein under these circumstances can assemble into filamentous structures. Double-label immunofluorescence studies which combine monoclonal antibodies RD301 and RV202 with the pVim and pDes antisera, respectively, show that the vimentin containing filaments matched completely with the pVVim2 containing structures, strongly suggesting that pVVim2 protein copolymerizes with vimentin and becomes a part of the vimentin skeleton. This is in agreement with cross-linking studies in which copolymerization has been shown between vimentin and desmin [22, 28]. Since the pVVim2 protein contains the vimentin α -helical domain and the desmin carboxy-terminus, which is supposed to protrude from the skeletal backbone [9], copolymerization between pVVim2 and vimentin is not surprising.

Intermediate filament proteins are expressed in a developmentally regulated and tissue-specific fashion, and this pattern of expression has been conserved among vertebrate species. However, some differences which may relate to a slight divergence in the developmental pattern of a distinct cell lineage, have been noted [15]. This supports the view that intermediate filaments fulfill an important role in development. Furthermore, it suggests that alterations in highly conserved protein domains, which are supposed to be of crucial importance for proper functioning of IFs, are probably not tolerated. The amino acid sequence of the carboxy-terminal domain of vimentin is much more conserved among species than the sequence of the amino-terminal domain and approximates the conservation of the rod domain [34]. This is also observed for desmin [19]. However, the carboxy-terminal regions of different classes of IF subunits have significantly diverged. This is suggestive for the importance of the carboxy-terminal region in IF-specific function. Recently, it has been shown that the carboxy-terminal domain of both vimentin and desmin can associate with lamin B at the nuclear envelope [10, 11]. Our results show that the presence of significant levels of pVVim2 protein in all vimentin-expressing cells does not interfere with normal mouse development. Preliminary results using transgenic mice in which desmin is expressed in a vimentin-specific fashion also indicate that the expression of desmin in mesenchymal cells does not interfere with normal development. This suggests that, if vimentin fulfills an essential function in development, this function is not hampered by the coexpression of pVVim2 or desmin.

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CHAPTER 6

TRANSGENIC EXPRESSION OF THE MUSCLE-SPECIFIC INTERMEDIATE FILAMENT PROTEIN DESMIN IN NONMUSCLE CELLS

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ABSTRACT

The coding region of the hamster desmin gene was fused to the 5' flanking sequences of the hamster vimentin gene and introduced into the germ line of mice. The expression of this intermediate filament gene construct (pVDes) was analyzed at the RNA and protein level in transgenic mice as well as in fibroblast cell lines and primary hepatocyte cultures derived from these mice. In all transgenic mice, the pVDes-encoded protein was coexpressed with mouse vimentin in a tissue-specific fashion and was indistinguishable from normal hamster desmin. Culturing of transgenic hepatocytes induced desmin expression indicating that 3.2 kbp of the vimentin gene 5' region regulates both tissue-specific and tissue culture-induced intermediate filament protein expression. Immunohistochemical staining and double-label immunoelectron microscopy of cultured transgenic fibroblasts showed that the pVDes protein assembled into intermediate filaments which colocalized with the mouse vimentin filaments. Endogenous vimentin RNA levels were not influenced by high-level pVDes expression. The coexpression of desmin and vimentin in nonmuscle cells did not result in detectable developmental, morphological, or physiological abnormalities.

INTRODUCTION

Intermediate filaments (IFs) represent a unique group of cytoskeletal structures that occur in the cytoplasm of virtually all mammalian cells. The expression of the different classes of IF subunits is regulated in a tissue-specific and developmentally regulated fashion. Generally, cytokeratins are expressed in cells of epithelial origin while neurofilaments are expressed in neuronal cells, glial fibrillary acidic protein in astrocytes, desmin almost exclusively in striated and most smooth muscle cells, and vimentin mainly in cells of mesenchymal origin [58, 59, 64]. The expression of nuclear lamins, which are part of the IF multigene family, is also cell type-specific and developmentally regulated [12, 29, 61]. The highly conserved specificity of IF subunit expression during development suggests that each type of subunit plays an important role in cellular differentiation.

On the basis of gene structure and sequence data, the IF subunits have been divided into four subfamilies. Vimentin [47], desmin [48], glial fibrillary acidic protein [40], and possibly a recently described neurofilament protein [39] display a high degree of homology and constitute one subfamily of proteins (type III subunits). Like the other IF proteins, they have a conserved α -helical domain of ~310 amino acid residues, flanked by nonhelical amino-terminal and carboxy-terminal domains of variable length and sequence [12, 22, 58, 59, 64]. In view of the highly-specific expression pattern of each IF subunit, it may be assumed that their variable terminal regions at least partly determine the specific properties that are needed in different cell types during various stages of development [23, 38, 58].

The vimentin expression pattern is relatively complex. During embryogenesis, vimentin is the first of the nonepithelial subunits to be expressed [18, 30]. The appearance of the cell type-specific IF subunits is often preceded by vimentin expression, and in certain cases coexpression with the other types of IF is observed [9, 19, 37, 56, 57, 64, 65]. When non-mesenchymal cells are dissociated from tissues and brought into culture, induction of vimentin synthesis often occurs [15, 64]. This is mostly accompanied by the continued coexpression of the tissue-specific IF protein, but in some cases only vimentin expression remains [6, 17]. The level of vimentin expression is growth regulated and can be stimulated by some growth factors [11, 54]. This complex expression is mediated by multiple regulatory elements in the 5' flanking sequence of the vimentin gene [46, 54].

In cultured cells, the study of cell type-specific regulation and function of IF expression is necessarily limited. The phenomenon of tissue culture-induced vimentin expression is not understood. The observations, that some cell lines do not express IFs [27, 41, 64], and

that disruption of IFs does not affect growth or morphology of cultured cells [21, 34, 42], indicate that IFs -at least *in vitro*- do not fulfill an essential cellular function [13]. Transgenic mice, which have proven to be useful in the study of tissue-specific gene expression [for review see: 44], provide a better system to study IF gene regulation and function. Our approach was to change the normal IF expression pattern by introducing an IF gene construct into the germ line of mice composed of the 5' flanking region of the vimentin gene and the complete coding region of the desmin gene. Transgenic mice, primary cell cultures, and cell lines derived from these mice were analyzed for the pattern and levels of desmin and vimentin expression.

MATERIALS AND METHODS

Plasmid construction

For construction of the pVDes gene, the 0.7 kbp HpaII-BamHI fragment from the hamster desmin gene (from +25 bp to +700 bp relative to the cap site [48]) was subcloned into an AccI-BamHI-digested pUC19 plasmid. The 3.2 kbp BamHI-EcoRI (from +700 to +3,900) and the 3.4 kbp EcoRI-StuI (from +3,900 to +7,300) desmin fragments were ligated immediately 3' to this fragment, thereby generating a complete desmin gene without 5' sequences upstream of the HpaII site at +25 bp. In this construct, 60 bp of 5'- and 775 bp of 3' untranslated sequences (including the "poly A" signal) are present. The 3.2 kbp vimentin promoter region (ranging from -3,100 to +101 relative to the vimentin cap site) was isolated as a HindIII fragment by ligating the 3.45 kbp BamHI-EcoRI fragment from p3.1VimCAT [46] into an EcoRI-BamHI-digested pUC19 plasmid, and subsequent partial HindIII digestion of this clone. This vimentin 5' region was ligated into the HindIII site of the pUC19 polylinker of the complete desmin gene in the 5'-3' orientation. The resulting construct (pVDes) contains some additional base pairs from the pUC19 polylinker between the HindIII and the AccI site. To facilitate removal of plasmid sequences before microinjection, the BamHI site in the first intron of desmin was removed by filling in and subsequent blunt end ligation after partial BamHI digestion.

Generation and identification of transgenic mice

Transgenic mice were generated by pronuclear microinjection as described previously [36]. Plasmid sequences were removed by BamHI (5', complete) and EcoRI (3', partial) digestion, leaving a 10.5 kbp IF hybrid gene. This was isolated and purified by preparative gel electrophoresis and electroelution, dissolved in ultrapure water, and dialyzed against 10 mM Tris-HCl (Merck, Darmstadt, FRG), 0.1 M EDTA (Merck), pH 7.4. The DNA concentration was adjusted to 4 $\mu\text{g} \cdot \text{ml}^{-1}$, and ± 200 pVDes copies were injected. Several weeks after birth of animals that had developed from microinjected eggs, tail DNA was isolated and analyzed by Southern blotting. Briefly, equal amounts (10 μg) of total genomic DNA of each mouse was digested with the appropriate restriction enzyme(s), run on a 0.6% agarose gel, and transferred to nitrocellulose. The filter was hybridized to ^{32}P -labelled probes (see below) and washed as described [5]. The number of integrated pVDes copies was determined by Southern blot analysis of serial dilutions of tail DNA, using the vimentin-specific probe E49, and subsequent densitometric scanning of the autoradiographs. pVDes hybridization signals were compared to those of the single copy mouse vimentin gene of serial dilutions of the pVDes plasmid.

Northern blot analysis and hybridization probes

Total cellular RNA was isolated from tissues and cultured cells by the LiCl-urea method [1]. Primer extension analysis on total cellular RNA from ear fibroblast cell lines was performed as described [24 (procedure 2)], using a 21-mer 5' desmin primer (5'-GGAGGCAGCGGGCAGGCAGCCC-3'; from +25 to +46 relative to the transcription initiation site). For Northern blotting, 10, 15, or 20 μ g RNA samples were glyoxylated, fractionated on a 1.2% agarose gel, and transferred to Highbond-N (Amersham International, Amersham, UK). Hybridization was performed as described [5]. A 350 bp hamster desmin Sau3A fragment in M13 (X54), covering a region from 25 bp of exon 8 to 120 bp into the 3' untranslated region, was used as desmin probe [46, 48]. A 520 bp hamster vimentin Sau3A fragment in M13, ranging from -150 to +370 bp relative to the cap site, was used as vimentin probe [46, 47]. As an actin probe, we used a 1.25 kbp PstI hamster actin cDNA fragment [8], which hybridizes to α -, β -, and γ -actin. Densitometric scanning was performed on autoradiographs exposed for different times.

Cell culture

Ear fibroblast cell lines were established and cultured as described [3].

Mouse hepatocytes were isolated and cultured using the hepatic portal perfusion method as described [32, 33], with the following modifications. Instead of Hank's solution, Williams E medium (Flow Laboratories Inc., McLean, VA, USA) supplemented with 2.5 mM EGTA (Merck) was used. Hepatocytes were cultured in Williams E medium supplemented with 10% fetal calf serum (FCS; Flow Laboratories), 2mM L-glutamine (Flow Laboratories), 20 mU.ml⁻¹ bovine insulin (Flow Laboratories), 1 μ M dexamethasone (Flow Laboratories), and antibiotics (25 μ g.ml⁻¹ fungizone, 100 μ g.ml⁻¹ vancomycin, and 50 μ g.ml⁻¹ gentamycin (all obtained from Flow Laboratories)).

Gel electrophoresis and immunoblotting

Cytoskeletal preparations of eye lens and cultured cells were obtained as described [35]. One- (1D) and two-dimensional (2D) SDS-gel electrophoresis and immunoblotting procedures have also been described previously [4].

Immunohistochemical analysis

Single- and double-label indirect immunofluorescence analyses of frozen tissue sections and cultured cells were performed as described previously [36]. In the underlying study, however, muscle tissue sections were pretreated with 0.5% Triton X-100 (BDH Chemicals Ltd., Poole, UK) in PBS before incubation with the first antibodies. The following polyclonal and monoclonal antibodies were used:

1. An affinity-purified polyclonal rabbit antibody (pKer) raised against human skin keratins, which reacts with virtually all epithelial tissues [51].
2. An affinity-purified polyclonal rabbit antibody (pVim) to vimentin [51].
3. A polyclonal rabbit antibody (pDes) to desmin [52].
4. The monoclonal antibody RV202 to vimentin [4].
5. The monoclonal antibody RD301 to desmin [4].
6. The monoclonal antibody RCK102 to human cytokeratins 5 and 8 [53].

Figure 1: A) Schematic representation of the IF gene construct pVDes (not drawn to scale). The line on the left represents the vimentin gene 5' flanking region (from -3,100 to +101 relative to vimentin cap site). Bar represents the desmin gene with black regions indicating the desmin 5' and 3'-untranslated sequences, hatched regions indicating exons, and open regions indicating the introns. Positions of the vimentin-specific (E49) and desmin-specific (X54) probes are indicated. E: EcoRI restriction site; B: BamHI restriction site. B) Southern blot analysis of tail DNA from mice transgenic for pVDes. $\pm 10 \mu\text{g}$ of DNA was digested with EcoRI. Hybridization was performed with the vimentin probe E49. Asterisk indicates position of the 8.5 kbp EcoRI fragment from the single copy mouse vimentin gene. Arrow indicates position of the 5.15 kbp EcoRI fragment from pVDes. lane 1) DNA from offspring of founder 430; lane 2) DNA from founder 430; lanes 3 and 7) DNA from control mice; lanes 4 and 5) DNA from offspring of founder 426; lane 6) DNA from founder 426; lanes 8 and 9) DNA from offspring of founder 429; lane 10) DNA from founder 429.

Transformed fibroblasts from control and transgenic mice were grown on Thermanox coverslips (Lux, Lab-Tek Div., Miles Laboratories Inc., Naperville, IL, USA), coated with fibronectin by a 30 min incubation with a crude preparation from human serum. Cells at $\pm 75\%$ confluency were washed with double Hanks' buffer and converted to cytoskeletons by extraction for 2.5 min at room temperature with a buffer (pH 7.2) containing 0.5% Triton X-100 [63], and fixed in buffered 0.5% paraformaldehyde (Merck) containing 0.3% Triton X-100 (pH 7.2). The cells were processed for immunogold labelling and electron microscopy essentially as described by De Mey [7], and as in AuroProbe EM product information (Janssen Pharmaceutica, Beerse, Belgium). To reduce background staining the coverslips were preincubated for 20 min with a TBS solution (pH 8.2) containing 10% normal goat serum and 0.1% BSA. The monoclonal and polyclonal antibodies were diluted in a similar buffer containing 1% normal goat serum and 0.1% Tween20 (Sigma Chemical Company, St. Louis, USA). Washing buffers also contained 0.1% Tween20. Furthermore, the 5 nm and 10 nm antibody-coated colloidal gold preparations (Janssen Pharmaceutica) were preabsorbed extensively on fibroblast cytoskeletons and diluted in Tris-buffer, pH 8.2, containing 1% BSA.

RESULTS

Generation of transgenic mice

We have demonstrated previously that the structural gene for hamster desmin and modifications thereof can be expressed after gene transfer into different types of cultured nonmuscle cells resulting in assembly of newly synthesized protein into intermediate filaments [28, 48]. Regulatory sequences which control desmin expression are

Figure 2: Expression of desmin and vimentin in tissues from control and transgenic mice as detected with the indirect immunofluorescence assay using pDes and pVim. a-c) Tail skin from control (a, b) and transgenic (c) mice incubated with pVim (a) and pDes (b, c); E: epithelial tissue; H: hair follicle; d-f) Esophagus from control (d, e) and transgenic (f) mice incubated with pVim (d) and pDes (e, f). Arrow indicates desmin-positive Langerhans cells; M: muscle tissue; E: epithelial tissue; g and h) Cells from blood smears of transgenic mice showing a filamentous staining pattern after incubation with pDes; i-k) striated muscle tissue of control (i, j) and transgenic (k) mice incubated with pVim (i) and pDes (j, k).

Note staining of transgenic muscle and nonmuscle cells with pDes; l and o) Testis tissue from control (l, m) and transgenic (n, o) mice incubated with pVim (l, n) and pDes (m, o). Note presence of desmin only in myoid cells and blood vessel smooth muscle cells in both control and transgenic testis tissues. Other tissues investigated include kidney, liver, spleen, heart, brain, tongue, bladder, uterus, and prostate. Bars indicate 4 μ m.

located in a region between -89 and +25 relative to the transcription initiation site [46]. We removed these regulatory elements by deleting the complete 5' flanking region of the desmin gene up to the HpaII site at position +25, and replaced it by 3.2 kbp of 5' flanking sequences (-3,100 to +101) from the hamster vimentin gene (Fig. 1A). This upstream region contains regulatory elements that confer high levels of vimentin expression in cultured cells and are instrumental in the down-regulation of vimentin expression during myogenesis [46]. The resulting IF gene construct pVDes (10.5 kbp without plasmid sequences) consists of the complete coding region of the desmin gene and additional 5' and 3' untranslated regions, fused to 3.2 kbp of vimentin upstream sequences (Fig. 1A; see Materials and Methods).

After removal of plasmid sequences the pVDes gene was injected into the most accessible pronucleus of fertilized mouse eggs. Southern blot analysis of tail DNA of mice born from these manipulated eggs showed that 5 of 32 mice had incorporated multiple copies of the construct into their genome in a head-to-tail tandem array. Three of these (strains 426, 429 and 430) produced offsprings, all of which inherited the pVDes transgene (Fig. 1B). Southern blot analysis revealed no detectable rearrangements in the pVDes sequences (data not shown). Offspring from founders 426 and 429 contained ~25 and 30 copies, respectively, whereas 430 and its offspring had incorporated only 3 pVDes copies. F₁ and F₂ analysis showed that integration had taken place into single loci.

Tissue-specific pVDes expression

The desmin expression pattern in tissue sections from transgenic mouse strains 426, 429 and 430 and from control mice was analyzed by the indirect immunofluorescence technique, using polyclonal rabbit antisera directed against desmin (pDes), vimentin (pVim), and cytokeratins (pKer) [51, 52]. From transgenic mice 427 and 428 only tail sections were examined. From transgenic mouse strains 426, 429 and 430 a number of tissues were analyzed and for each of these strains at least two male and female mice were used. Cells expressing vimentin but not desmin in tissues from control mice (e.g., fibroblasts, endothelial cells, cartilage, Kupffer cells, macrophages, Schwann cells) displayed intensive staining in transgenic tissues after incubation with pDes and with pVim (Fig. 2). Occasionally, a filamentous staining pattern was observed, suggesting that the pVDes-encoded desmin had assembled into IFs (Fig. 2g, h). Epithelial tissues stained with pKer but not with pDes. In transgenic striated muscle tissue, connective tissue fibroblasts not only expressed vimentin (Fig. 2i) but also desmin, both in skeletal (Fig. 2k) and heart muscle (not shown). All five transgenic mice and their offspring (from 426, 429 and 430) expressed pVDes in a vimentin-specific fashion. Surprisingly, in testis from the three transgenic mouse strains, Sertoli and Leydig cells, although expressing vimentin, deviated from all other tissues tested (listed in legend to Fig. 2) in that they did not contain detectable amounts of desmin (Fig. 2l-o).

We did not observe any morphological effects of pVDes expression, nor did the transgenic mice show detectable developmental or physiological abnormalities.

Characterization of *in vivo*-synthesized pVDes protein

To identify biochemically and characterize the *in vivo*-synthesized pVDes protein, we analyzed eye lens extracts from transgenic mouse strains 426, 429 and 430. Eye lens tissue has the advantage of expressing high levels of vimentin in the absence of desmin [50]. 1D SDS-gel analysis of total lens extracts (Fig. 3A) and cytoskeletal fractions (not shown), followed by immunoblotting with monoclonal antibodies to desmin (RD301) and vimentin (RV202), showed that the pVDes protein is expressed in transgenic lenses (Fig. 3A, B). Immunoblotting of 2D gels from total lens extracts (Fig. 3C) confirmed the

Figure 3: Characterization of pVDes protein from eye lens. A) Coomassie Brilliant Blue (CBB)-stained SDS-polyacrylamide gels with equal amounts of total lens extracts from 1) control and transgenic mice: 2) strain 426, 3) strain 429 and 4) strain 430. B) Immunoblots on total lens extracts from control and transgenic mice incubated with the monoclonal antibody to desmin (RD301; bmk, 1-4) and then with the monoclonal antibody to vimentin (RV202; bmk', 1'-4'). Cytoskeletal proteins from BHK-21/C13 cells were used as markers. C) Total lens extracts of control (1, 1') and transgenic mice from strains 429 (3, 3') and 430 (4, 4') analyzed by 2D SDS-gel electrophoresis and immunoblotting. Blots were incubated with RD301 (1, 3, 4) and then with RV202 (1', 3', 4').

identity of the pVDes protein, which comigrated with desmin from BHK-21/C13 hamster cells (not shown). Eye lenses from strains 426 and 429 contain comparable amounts of desmin. A relatively low concentration of hamster desmin was detected in strain 430 lenses. As judged from Coomassie Brilliant Blue-stained gels and immunoblots of both total lens extracts and cytoskeletal fractions, lens vimentin expression levels were not influenced by pVDes expression.

In vivo expression levels of pVDes

To determine the pVDes mRNA expression levels in different tissues and its influence on endogenous vimentin and desmin expression, RNA from transgenic and control tissues was analyzed by Northern blotting. We also included RNA from transgenic mice which express the vimentin-desmin hybrid gene pVVim2 [36]. In this construct, the last three exons of the vimentin gene have been replaced by the last three exons of the desmin gene, allowing the detection of the pVVim2 transcript with a desmin-specific probe. Blots were first hybridized to a vimentin-specific probe (E49; Fig. 1A), which recognizes both endogenous vimentin, pVDes and pVVim2 transcripts, and subsequently to a desmin-specific probe (X54; Fig. 1A). In control tissues only a 2.0 kbp mRNA band was detected, representing the mouse vimentin messenger (e.g., Fig. 4A, lane 4). In transgenic tissues an additional 2.4 kbp or 2.3 kbp band was observed that corresponds to the correctly sized pVDes transcript (e.g., Fig. 4A, lanes 1-3), or the pVVim2

Figure 4: Northern blot analyses of 15 μ g of total RNA from skeletal muscle (A), heart (B), and testis (C) from control and transgenic mice. Blots were hybridized to a vimentin specific probe (E49; Vim), and then to a desmin-specific probe (X54; Des). The minor additional band above the desmin mRNA position was observed in all tissues and probably represents a splicing intermediate or is derived from a cryptic initiation site. After dehybridization the blots were hybridized to an actin probe (Act) that recognizes both α -actin (1.8 kbp) and β/γ -actin transcripts (2.1 kbp). Similar analyses were performed on RNA from kidney, liver, spleen, brain and lens (results not shown). A) RNA from skeletal muscle of abdomen (lanes 1, 3, 4, 5) and thigh (lanes 2, 6). lanes 1, 2 and 8) pVDes strain 426; lanes 3) 429; lanes 4) control; lanes 5-7) pVVim2 strain 44. To illustrate the difference in length between pVVim2 and pVDes transcripts (2.3 and 2.4 kbp, respectively), RNA from strains 44 and 426 was run on a

longer gel and hybridized to E49 (lanes 7, 8). B) RNA from heart tissue. lanes 1) pVDes strain 426; lanes 2) 429; lanes 3) control; lanes 4) pVVim2 strain 44. To identify positively pVDes and endogenous desmin mRNA, longer exposure times, low stringency washes, and longer gels were used (lanes 2' and 3'); lane 2') as for lane 2, hybridized to E49; lane 2'') as for lane 2', hybridized to E49 and X54, subsequently; lane 3') as for lane 3, hybridized to E49 and X54, subsequently; lanes 1-4) exposure time and gel length as in figure 4A and C. C) RNA from testis. lanes 1) pVDes strain 426; lanes 2) 429; lanes 3) control; lanes 4) pVVim2 strain 44. After hybridization to X54 and long exposure times, a faint 2.3 kbp band was observed in lanes 1-3, representing endogenous desmin (not shown).

Figure 5: A) Double-label immunofluorescence staining of control (a and b) and transgenic (c and d) transformed ear-shell fibroblasts incubated with pVim (a, c) and the monoclonal antibody to desmin RD301 (b, d). Note colocalization of filaments stained by both antibodies in transgenic ear-shell fibroblasts. Bars indicate 4 μ m. B) Colocalization of desmin and vimentin in IFs of transgenic cultured ear-shell fibroblasts as seen at the ultrastructural level after double-immunogold labelling. All preparations were incubated with a mixture of secondary antibody-coated colloidal gold probes in the detection step. a) IFs from transgenic fibroblasts incubated with PBS instead of primary antibodies. b and c) IFs from control (b) and transgenic (c) fibroblasts, incubated with pVim and RD301, detected with 50 nm and 10 nm gold particles, resp. d and e) IFs from transgenic fibroblasts, incubated with pDes and RV202, detected with 5 nm and 10 nm gold particles, respectively. Bar indicates 200 nm.

Figure 6: Northern blot analysis of 10 μ g of total RNA from exponentially growing control (C) and transgenic transformed ear-shell fibroblast cell lines (pVDes strains 426, 429, and 430; pVVim2 strain 32). To exclude clonal variation and influences of growth conditions on expression levels, RNA was isolated from two independent cell lines for each pVDes strain. Blots were hybridized to a desmin- (A, X54; Des) and subsequently to a vimentin-specific probe (B, E49; Vim). After dehybridization the blots were hybridized to an actin-specific probe (Act). Fibroblasts from strain 430 contained relatively little pVDes transcripts, which were readily detected, however, after longer exposure times (lanes 430').

transcript, respectively (e.g., Fig. 4A, lanes 5 and 6). Primer extension analysis showed that transcription started at the authentic initiation site (data not shown). Expression levels were determined by densitometric scanning of autoradiographs from blots containing equal amounts of RNA. Actin mRNA levels served as a standard. The levels of vimentin mRNA did not differ between control and transgenic tissues (Fig. 4). Relative to mouse vimentin mRNA, the amount of pVDes transcripts varied between different tissues. In skeletal muscle tissue, pVDes expression was relatively high, exceeding mouse vimentin transcript levels (Fig. 4A). Both pVDes and pVVim2 mRNA levels were identical for skeletal muscle tissue from different parts of the body (Fig. 4A, lanes 1, 2, 5, 6). The lowest pVDes expression levels were detected in heart tissue (Fig. 4B). Again, no pVDes expression was observed in testis from different transgenic strains (Fig. 4C) indicating that the lack of expression in Sertoli and Leydig cells is caused at the level of transcription or as a result of mRNA instability. In contrast, pVVim2 was expressed at high levels in testis of transgenic mice (Fig. 4C, lane 4). Generally, pVDes expression levels were higher in tissues from strain 429 than in corresponding tissues from strain 426 (data not shown). An exception is skeletal muscle tissue, which in strain 426 contained almost twice the amount of pVDes mRNA found in strain 429 (Fig. 4C). The relative levels of pVVim2 expression also varied between different tissues and were highest in skeletal muscle.

***In vitro* expression of pVDes**

Transgenic cell lines were established by immortalization of ear-shell fibroblasts from offspring of strains 426, 429 and 430 with SV40. Double-label immunofluorescence assays showed that in each of these cell lines both desmin and vimentin are expressed and assembled into filamentous structures (Fig. 5A). In assays where the polyclonal vimentin antiserum and the monoclonal antibody to desmin were combined, vimentin and desmin staining intensities were about equal for cell lines 426 and 429, whereas cells from line 430 showed a much weaker desmin staining intensity. Upon incubation of line 430 fibroblasts with pDes, this difference was not observed. Generally, there was a complete match of desmin and vimentin filaments (Fig. 5A: c, d). Double-immunogold labelling of control and transgenic fibroblasts revealed that the pVDes protein coassembled into bona fide IFs together with endogenous vimentin (Fig. 5B, a-e).

The pVDes and vimentin expression levels of the transgenic cell lines were analyzed by Northern blot analysis using desmin- and vimentin-specific probes (Fig. 6A, B). The pVDes transcript was of the expected size (2.4 kbp) and was detected in all three cell lines (Fig. 6A). For cell line 430, the pVDes mRNA level was 20-fold lower than that of mouse vimentin mRNA. In contrast, cell lines 426 and 429 showed pVDes mRNA levels that were similar to those of the endogenous vimentin. The amount of vimentin mRNA (2.0 kbp) was not influenced by pVDes expression (Fig. 6B). This was also observed for a pVVim2 expressing ear-shell fibroblast cell line (strain 32; [36]), which was cultured in parallel (Fig. 6B). As in transgenic tissues, pVDes mRNA was less abundant than pVVim2 mRNA.

To identify the pVDes protein in the ear-shell fibroblast cell lines and determine the correlation between transcript and protein levels *in vitro*, the relative amounts of IF protein in cytoskeletal fractions of the transgenic cell lines were analyzed by 1D SDS-gel electrophoresis (Fig. 7).

Desmin and vimentin were identified by immunoblotting with monoclonal antibodies to desmin (RD301) and vimentin (RV202), subsequently (Fig. 7B). The pVDes protein has an apparent molecular mass of 54 kDa and cannot be distinguished from hamster desmin of BHK-21 cells. Coomassie Brilliant Blue-stained gels reflected the levels of IF mRNA (Fig. 6) at the protein level (Fig. 7A).

Induction of pVDes expression in tissue culture

In liver tissue, hepatocytes express specific cytokeratins and completely lack vimentin [16]. Culturing of rat hepatocytes induces vimentin expression [17]. Indirect immunofluorescence assays with pVim, pDes and pKer on frozen sections of liver from control and transgenic mice confirmed that hepatocytes express only cytokeratins, whereas Kupffer cells express vimentin in control mice and also desmin in transgenic mice.

Figure 7: Cytoskeletal fractions of control (lanes 1) and transgenic (lanes 2-4) transformed ear-shell fibroblast cell lines, analyzed by 1D SDS-gel electrophoresis and immunoblotting. A) Coomassie Brilliant Blue (CBB) staining of SDS-polyacrylamide gel. B) Immunoblots, incubated with the monoclonal antibody to desmin (RD301, mDes) and subsequently with the monoclonal antibody to vimentin (RV202, mVim). lane 1) Control fibroblasts; lane 2) strain 426; lane 3) strain 429; lane 4) strain 430.

Figure 8: Indirect immunofluorescence microscopy of primary hepatocyte cultures from control mice. Staining was performed with monoclonal and polyclonal antibodies to desmin, vimentin, and cytokeratins after different periods of culture. a) Hepatocytes cultured for 8 h, pVim stained. Note absence of vimentin filaments in hepatocytes and strong, filamentous staining of a contaminating fibroblast. b and c) Double-label immunofluorescence staining for vimentin and cytokeratin (pVim and RCK102, respectively) after 8 h of culture. Hepatocytes are vimentin negative (b) but contain cytokeratin filaments (c). d and e) Hepatocytes cultured for 18 h and 40 h, respectively, and pVim stained. Note gradual increase in vimentin expression. f and g) Double-label immunofluorescence staining for vimentin and cytokeratin (pVim and RCK102, respectively) after 40 h of culture. Hepatocytes contain both vimentin (f) and cytokeratin filaments (g), which do not seem to colocalize. Bars indicate 4 μ m.

Figure 9: Indirect double-label immunofluorescence staining of primary hepatocyte cultures from pVDes transgenic mice, strains 429 (a-d) and 426 (e and f). a and b) Staining for desmin (a; pDes) and cytokeratin (b; RCK102) after 35 h to 40 h of culture. Note filamentous desmin staining of one hepatocyte with pDes while an adjacent hepatocyte is completely desmin negative (a). Both cells express cytokeratins in large amounts (b). c and d) Staining for vimentin (c; pVim) and desmin (d; RD301). Note expression of both vimentin (c) and desmin (d). e and f) Staining for vimentin (e; pVim) and desmin (f; RD301) of strain 426 hepatocytes after 70 h of culture. Note complete absence of desmin staining (f), while vimentin is expressed abundantly (e). Bars indicate 4 μ m.

Hepatocytes from control and transgenic mice (strains 426 and 429) were isolated, cultured as a monolayer, and analyzed for IF expression by the indirect immunofluorescence assay during 4 days of culture. In all cases, the onset of vimentin synthesis was observed after 12-14 h (Fig. 8). At this point, vimentin expression occurred in only few cells and staining intensities were low. In addition, the filaments were not distributed uniformly throughout the cell but were located at the periphery (Fig. 8). Within the next 30-40 h, the number of vimentin-containing cells and the amount of vimentin in each cell increased until almost every cell was vimentin positive. Cytokeratins were expressed continuously and did not colocalize with vimentin filaments (Fig. 8f, g). About 25 h after the earliest detection of vimentin in the immunofluorescence assays, hepatocytes from transgenic mice of strain 429 initiated expression of desmin (Fig. 9a, b). Again, a gradual increase in expression over a 40-hour period was observed until most cells were desmin positive. In control hepatocytes, desmin expression was never observed. Eventually, desmin and vimentin staining patterns were identical, suggesting copolymerization of these proteins. In cultured hepatocytes from four different transgenic mice of strain 426, desmin expression could not be detected (Fig. 9e, f). Each of these mice, however, showed the tissue-specific expression pattern that is characteristic of all pVDes transgenic mice and, as described, pVDes levels in strain 426 were generally comparable to those of strain 429, both in tissues and in the fibroblast cell lines. To extend our analysis, hepatocytes of the two different pVVim2 transgenic mouse strains (Nos. 32 and 44; [36]) were also brought into culture. Hepatocytes from these mice displayed vimentin and cytokeratin expression patterns, which did not differ from those of control and pVDes hepatocytes. Initiation of pVVim2 expression was observed 1 day later than vimentin expression.

In summary, tissue culture-induced IF gene construct expression occurred in three out of four transgenic strains.

DISCUSSION

Expression of desmin in adult mammalian tissues occurs almost exclusively in muscle cells. We have changed the tissue-specific distribution of this IF protein by creating transgenic mice, which express the IF gene construct pVDes, composed of the coding region of the hamster desmin gene and the 5' flanking region of the vimentin gene, in a vimentin-specific fashion. The pVDes protein was indistinguishable from hamster desmin. The levels of pVDes protein recovered in cytoskeletal fractions from transgenic cell lines correlated with the pVDes mRNA levels, supporting the notion that IF synthesis is determined primarily by the amount of mRNA present [43, 46, 54, 58]. Double-label immunofluorescence assays and double-immunogold labelling of cultured transgenic ear-shell fibroblasts showed that the pVDes protein assembled into IFs that colocalized with the endogenous vimentin filaments. This is in agreement with previous reports concerning coexpression of desmin and vimentin in different types of muscle cells (49, 62). Taken together, the data indicate that the pVDes-encoded desmin subunits are incorporated into the endogenous vimentin filaments.

The *in vivo* expression pattern of the pVDes protein was determined by immunohistochemical analysis of tissue sections from different transgenic strains. Each transgenic mouse and all offspring expressed hamster desmin in a vimentin-specific fashion. pVDes expression did not detectably affect cellular morphology or differentiation. Desmin synthesis in vimentin-negative cells was never observed. Surprisingly, no pVDes expression was detected in Sertoli and Leydig cells of all three lines of transgenic mice although these cells do express vimentin [12, 66, 67] (Fig. 2). This lack of expression is either caused at the transcriptional level or by pVDes mRNA instability since no pVDes

transcripts were detected in transgenic testis (Fig. 4C). In contrast, an IF gene construct (pVVim2), consisting of the 5' flanking region and first six exons plus introns of the vimentin gene, fused to the last three exons and introns of the desmin gene is expressed in these testicular cells. Since regulatory elements have been identified within introns of some genes [2, 26, 35, 45, 55, 60], it is possible that such elements are located within the first six introns of the vimentin gene.

An important aspect of gene regulation in general is the establishment of the appropriate level of expression. For vimentin, questions concerning the mechanisms of this aspect of regulation cannot be addressed by the use of cultured cells only, since it is unclear whether or not expression levels are elevated upon culturing of vimentin-expressing cells, overruling regulatory mechanisms active *in vivo*. We show here that endogenous vimentin mRNA and protein levels were not influenced by pVDes expression. Obviously, the amounts of IF protein are not strictly regulated and can be elevated without deleterious effects.

The highest pVDes transcript levels were detected in skeletal muscle tissue (Fig. 4). Immunohistochemical staining of striated muscle tissue sections showed that nonmuscle cells, which are included in this tissue, express both vimentin and pVDes (Fig. 2). Transgenic myoblasts presumably also express these proteins. Since the pVDes encoded hamster desmin is indistinguishable from endogenous mouse desmin in these stainings, we cannot exclude the possibility that pVDes is also expressed in muscle cells of transgenic mice; e.g., as a result of the presence of muscle-specific regulatory sequences within the introns of the desmin gene in addition to those present in the desmin gene 5' region [46]. However, pVVim2 mRNA levels were also highest in skeletal muscle tissue (Fig. 4), but it has been shown that the pVVim2 protein is not expressed in transgenic striated muscle cells [36]. Furthermore, in transgenic heart tissue pVDes mRNA levels were low although endogenous desmin is expressed at high levels (Fig. 4B).

Our studies on cultured hepatocytes establish that sequences in the 3.2 kbp of 5' flanking region of the vimentin gene confer tissue culture-induced vimentin expression. In hepatocytes from pVDes strain 429, desmin protein expression was first observed after 40 to 50 h of culture, and lagged ~25 h behind the initiation of vimentin synthesis. A similar time lag was observed in hepatocytes from pVVim2-expressing mice. pVDes and pVVim2 transcripts also were detected ~1 day later than vimentin mRNA (data not shown). This lapse may result from differences in mRNA stabilities. On the other hand, species differences in *cis* regulatory sequences of the hamster and mouse vimentin genes could explain the observed delay in desmin synthesis. Reports on vimentin expression during avian and mammalian hematopoiesis have shown that divergence of *cis*-linked sequences can result in species-specific regulation of expression [43]. Timing of human neurofilament (NF-L) gene expression in transgenic mice also differed from endogenous NF-L expression [31].

Cultured hepatocytes from pVDes strain 426 expressed vimentin, but no pVDes expression could be detected by indirect immunofluorescence (Fig. 9) and Northern blotting (data not shown). However, mice from this strain displayed tissue-specific pVDes expression at similar levels as strain 429. The site of integration of the pVDes copies in strain 426 might not allow transcriptional activation under *in vitro* conditions. At the same time, regulatory processes in control of tissue-specific and tissue culture-induced vimentin expression may not be identical. We did not detect major rearrangements in the vimentin promoter region of the pVDes copies of strain 426. It remains to be established exactly which 5' sequences mediate induction of vimentin synthesis in cultured cells.

The reasons for the existence of the highly-specific expression patterns of desmin and vimentin are unknown. Although the functional differences between these proteins are obscure, some different properties have been noted. Comparison of hamster vimentin and desmin *in vivo* phosphorylation patterns have shown that desmin contains an additional, subunit-specific phosphorylation site in the COOH-terminal "tail" region [10, 20]. This might allow a differential response to exogenous stimuli and may reflect subunit-specific

functions. In addition, it has been described that the "tail" domains of desmin and vimentin both associate with lamin B at the nuclear envelope but with different affinities [25]. In double-label immunofluorescence assays on transgenic ear-shell fibroblast cells and cultured hepatocytes we observed a small percentage of cells that displayed large differences in desmin and vimentin staining intensities, suggesting that some filaments contained predominantly desmin or vimentin subunits. The same observation has been made for pVVim2-expressing cultured fibroblasts [36]. This might reflect differences in assembly characteristics.

The coexpression of desmin and vimentin in tissues and cultured cells from transgenic mice did not result in detectable abnormalities. Future experiments, aimed at interfering with IF expression *in vivo* will hopefully shed some light on the function of these cytoskeletal structures.

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CHAPTER 7

DISRUPTION OF INTERMEDIATE FILAMENTS BY EXPRESSION OF DOMINANT NEGATIVE DESMIN MUTANTS IN TRANSGENIC MICE

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Summary of two manuscripts in preparation:

- Pieper FR, Schaart G, Raats JMH, Henderik JB, Ramaekers FCS, Berns A, Bloemendal H. Disruption of vimentin intermediate filaments in transgenic mice by expression of a dominant negative mutant desmin subunit.
- Raats JMH, Schaart G, Pieper FR, Van de Kemp A, Berns A, Henderik JB, Ramaekers FCS, Bloemendal H. Muscle-specific expression of a truncated desmin subunit in transgenic mice.

ABSTRACT

To investigate the functions of desmin and vimentin intermediate filaments in the context of intact tissues and the developing organism, a construct encoding a truncated desmin subunit driven by the desmin or vimentin promoter (pDDV and pVDV, respectively), was introduced into the murine germ line. The mutant desmin was assembly-incompetent and capable of disrupting preexisting desmin and vimentin filaments in a dominant negative fashion, both in transgenic mouse tissues. In mouse tissues, high level expression of pDDV and pVDV occurred in a small percentage of desmin- or vimentin-containing cells, respectively, and also in skeletal muscle cells. Immunohistochemical staining of muscle tissue showed a diffuse desmin pattern instead of the dots and clumps into which mutant desmin typically accumulates in other cell types. The over-expression of pDDV and pVDV and the concomitant disruption of both the endogenous desmin and vimentin filaments in a significant percentage of cells as observed for pVDV did not cause any detectable developmental, morphological or functional abnormalities.

INTRODUCTION

The cytoskeleton of eukaryotic cells is a dynamic structure composed of microtubules, microfilaments, and intermediate filaments (IF). Single IFs are 10 nm in diameter, and have a length of approximately 40 μ m. IFs can comprise up to 85% of total cellular protein [6, 13, 19, 26, 27, 43, 44].

IF proteins are encoded by a large multigene family (> 35 genes) and can be divided into six different types on the basis of gene structure and sequence homology [6, 19, 26, 27, 44]. To a large extent, these different types of IF subunits are expressed in a developmentally regulated and tissue-specific fashion. This highly conserved specificity of expression would suggest that each type or combination of subunits plays a unique role in cellular differentiation [6, 13, 19, 26, 27, 43, 44]. Although all IF proteins have a highly conserved secondary structure, their physicochemical properties can be quite different.

While the function of microtubules and microfilaments in cellular processes is well understood, the function of IF proteins has long remained elusive. Cytoplasmic IF proteins clearly do not fulfill a household function, since several cell lines do not contain any cytoplasmic IF network. In addition, disruption of IF networks in cultured cells does not affect the cell morphology, motility or division [review: 19]. This has led to the assumption that the function of IF proteins must be manifested at the level of the tissue or organ as a whole, possibly in providing mechanical strength, cellular organization and architecture [15, 26, 30]. In agreement with this notion, vimentin IF proteins display unique viscoelastic properties allowing them to resist breakage and become even stronger under stress that would rupture other cytoskeletal networks [17].

To address the issue of IF function at this level, different approaches can be chosen. These include ectopic expression or over-expression of IF proteins in transgenic mice [1, 7, 8, 11, 18, 20, 25, 29], specific interference with *in vivo* IF assembly by microinjection of IF-specific antibodies [19] or anti-sense RNA into zygotes or developing embryos [47]. Also disruption of IF networks in transgenic mice or embryonic cells by expression of dominant-negative mutant IF subunits [8, 9, 15, 48, 51], and inactivation of IF genes via homologous recombination in embryo stem cells [Baribault 1991] has been applied to investigate IF function.

In cultured cells it has been demonstrated that IF networks can be disrupted in a dominant-negative fashion by expression of IF gene constructs containing modifications or deletions in the carboxy-terminal part of the central α -helical 'rod'-domain [2, 9, 16, 22, 25, 34, 35, 36, 42, 51, 53]. Interaction of the mutant- and wildtype IF subunits results in disassembly of preexisting IF networks and inhibits *de novo* IF formation.

Expression of keratin gene mutants in skin of transgenic mice caused disruption of endogenous keratin IF assembly and resulted in a pathological condition equivalent to the human skin disease epidermolysis bullosa simplex [9, 15, 51].

As an approach for studying the *in vivo* functions of desmin and vimentin, IF gene constructs based on the hamster desmin and vimentin promoters and a truncated hamster desmin gene were introduced into the murine germ line. It was previously shown that in transgenic mice expressing wildtype desmin in vimentin containing cells no developmental or morphological abnormalities could be detected, except for cataract formation [11].

Vimentin displays a complex expression pattern and is coexpressed with other IF subunits during various stages of development. When coexpressed with type III IF proteins (desmin, glial fibrillary acidic protein and peripherin), or type IV IF proteins (neurofilaments), coassembly of vimentin and the other IF subunits takes place [6, 13, 19, 44]. Hence, expression of the constructs described above is expected to affect IF networks containing type III IF subunits.

We show here that expression of a truncated desmin in transgenic mice results in disruption of desmin and vimentin IFs in a significant percentage of mesenchymal cells, without causing detectable developmental or morphological abnormalities.

MATERIALS AND METHODS

Plasmid construction

For construction of pDDV the 3.4 kb HincII-HincII hamster vimentin fragment [32] containing exon 9, the polyadenylation signal and 2.6 kbp of 3' flanking sequences, was cloned into a pUC19 digested plasmid. The 4.0 kbp EcoRI-Sall hamster desmin fragment [33] containing 3.4 kbp 5' flanking sequences, including the desmin promoter region, and

the 5' part of exon 1, and the 2.4 kbp Sall-XbaI hamster desmin fragment [33] containing the 3' part of exon 1 and exon 2, 3, 4, and 5, were ligated into the EcoRI-XbaI digested pUC19 vector containing the 3.4 kbp vimentin fragment. The resulting construct

pDDV contains some additional base pairs from the pUC19 polylinker between the XbaI and the HincII site (Fig. 1). For construction of pVDV, the 6.2 kbp XbaI fragment from pVDes [29] was subcloned into the XbaI site of pUC19, yielding pVDes-Xba. This

fragment comprises the 3.2 kbp hamster vimentin promoter and 3 kbp of the hamster desmin gene. The upstream XbaI site is part of a polylinker, the XbaI site in the desmin gene is located

in intron 5. A 3.4 kbp HincII fragment containing the hamster vimentin exon 9, the vimentin 3' untranslated region, the polyadenylation signal and 2.6 kbp of 3' flanking sequences [33] was sub-

cloned into SmaI polylinker site of pVDes-Xba immediately 3' of the XbaI site. Vimentin exon 9 contains 39 bp

Figure 1: Schematic representation of IF gene construct of pDDV and pVDV and of the resulting protein. Construct: black boxes represent desmin exons; white box: vimentin exon 9 (non-coding); striped box: coding region of vimentin exon 9; P indicates the desmin or vimentin promoter region of the pDDV or pVDV construct, respectively; L: ligation site; Xba: XbaI restriction site. IF protein: white bar represents helical 'rod' domain; stippled boxes: non-helical regions; black lines: non-helical 'head' and 'tail' end domains. Numbers refer to amino acid (aa) residues.

of coding sequences. As a result, pDDV and pVDV encode a protein of 353 amino acid residues, containing desmin amino acid residues 1-340 and vimentin amino acid residues 452-462 (Fig 1).

Generation and identification of transgenic mice

Transgenic mice were generated by pronuclear microinjection and subsequently identified by Southern blotting as described previously [20]. For pDDV containing mice plasmid sequences were removed by complete EcoRI-HindIII digestion leaving a 9.8 kbp carboxy terminally deleted desmin/vimentin hybrid gene. This gene was isolated and purified as described previously [29]. The DNA concentration was adjusted to 4 $\mu\text{g}.\text{ml}^{-1}$, and ± 200 pDDV copies were injected.

For pVDV containing mice plasmid sequences were removed by BamHI-EcoRI digestion leaving a 9.6 kbp truncated IF gene. Isolation and purification of this fragment were performed as described before [20, 29].

Mouse tail DNA was isolated and analyzed by Southern blotting several weeks after birth of animals that had developed from microinjected eggs.

Cell culture and transfection

Hamster lens cells [5], HeLa cells, and C2C12 muscle cells were transfected with the pDDV- or pVDV-construct using the calcium-phosphate precipitation method [28, 34, 52]. Cells were plated in 35 mm culture dishes (Costar Corp., Boston, MA, USA) 24-48 h before transfection and grown in DMEM (Flow Laboratories) supplemented with 10% fetal calf serum (FCS; Flow Laboratories). Transfections were carried out on cell cultures that had reached $\pm 40\%$ confluency. 5 μg Plasmid DNA was added to each culture dish as a calcium phosphate precipitate, 20 min later 2 ml culture medium containing 5 $\mu\text{g}.\text{ml}^{-1}$ chloroquine (Sigma Chemical Co, St. Louis, MO, USA) was added. After five hours of incubation the cells were glycerol shocked for 2.5 min, and incubated in normal growth medium for 48 h [28, 30, 34, 35]. Stable transfectants for pDDV were selected as described before [34].

To induce *in vitro* myotube formation of the C2C12 muscle cells, confluent cultures were switched to low mitogen fusion medium (DMEM supplemented with 2% horse serum; Flow Laboratories) [30].

Northern blot analysis

Northern blotting and hybridization were performed as described previously [39]. A mixture of PstI cDNA fragments derived from pVim [10] was used as a vimentin probe. As a desmin probe a 1.8 kbp HindIII-KpnI fragment was used, containing most of the hamster desmin cDNA [35]. A 520 bp hamster vimentin Sau3A fragment (E49) ranging from -150 to +370 relative to the transcription initiation site, was used as a probe to simultaneously detect vimentin and pVDV transcripts. As an internal standard the 1.25 kbp hamster actin cDNA fragment [10] was used. This actin probe hybridizes to α -, β -, and γ -actin. Probes were radiolabelled using the random priming method [14].

Immunocytochemistry and antibodies

Immunofluorescence staining procedures were performed on 5 μm thick cryostat sections of mouse tissues and on cultured cells as described before [20, 29]

The following antibodies were used in this study:

1. A polyclonal rabbit antiserum (pDes) to chicken gizzard desmin [40].
2. An affinity-purified polyclonal rabbit antiserum (pVim) to bovine lens vimentin [38].
3. The mouse monoclonal vimentin antibody RV202 [40].

Immunoblotting

Immunoblotting procedures were carried out as reported earlier [34, 41]. For preparation of IF fractions from mouse tissues, 20 μ m thick cryostat sections from fresh frozen mouse tissues were washed for 5 min in PBS and centrifuged for 10 min at 3,000 $\times$ g, followed by extraction in 0.5% Triton X-100 (BDH Chemicals Ltd., Poole, UK), 5 mM EDTA (Merck, Darmstadt, FRG), 0.4 mM PMSF (Merck) in PBS, pH 7.4, essentially as described [41]. After centrifugation, the cytoskeletal fractions were dissolved by boiling for 4 min in SDS-sample buffer [21] containing 2.3% sodium dodecylsulphate (SDS; BDH Chemicals Ltd.). Protein bands were visualized using horseradish-peroxidase conjugated swine anti-rabbit Ig (DAKO A/S, Glostrup, Denmark). Blots were stained with 4-chloro-1-naphthol (Merck) and 0.12% hydrogen peroxide (Merck). Cytoskeletal preparations of cultured cells were made by harvesting the cells from culture flasks, washing in PBS and extracted with 0.5% Triton X-100 as described above.

RESULTS

Modifications in the C-terminal region of the rod domain of the desmin subunit can render these subunits incapable of homopolymeric filament formation [35, 36]. Moreover, expression of the mutant desmin in vimentin containing cells can cause complete disruption of the endogenous vimentin and desmin IF networks [35, 36, 42].

To study the possible function of desmin in muscle cells, a dominant negative mutant desmin gene (pDDV) was constructed (Fig. 1) under control of 3.4 kbp noncoding 5' hamster desmin sequences. Within this 3.4 kbp region sequences are present which control desmin expression in myogenic cell lines [29, 24, 49]. The construct pVDV (Fig. 1) generated to investigate the *in vivo* and *in vitro* functions of vimentin, encodes a truncated desmin protein driven by the vimentin promoter. Therefore, analyses of tissues of transgenic mice expressing construct pDDV or pVDV would reveal whether the regions of the desmin- or vimentin promoter are sufficient for directing desmin expression in smooth, skeletal and heart muscle cells *in vivo* or vimentin expression in vimentin containing cells *in vivo*, respectively. The mutant encoded by the construct pDDV or pVDV, is truncated at amino acid 340, while the last 129 amino residues of desmin are replaced by the last 13 amino acids of vimentin (Fig. 1).

In vitro expression of pDDV and pVDV

To study the expression of pDDV and pVDV *in vitro*, immunofluorescence studies using polyclonal and monoclonal antibodies were performed on transiently or stably transfected cell lines: 1) HeLa cells, expressing both vimentin and keratins IF proteins. 2) Hamster lens cells express high levels of vimentin but no other cytoplasmic IF proteins. 3) the myogenic cell line C2C12, expressing vimentin and desmin.

pDDV-expression in vitro

In undifferentiated C2C12 cells stably transfected with the pDDV construct, an almost

Figure 2: Single- and double-label indirect immunofluorescence assay of control (a, b, f, g), as well as pDDV transfected (c, d, e, h, i), undifferentiated (a-e) and differentiated (f-i) C2C12 cells. Cells were incubated with the polyclonal desmin antibody (a, c, f-i), double-labelled with the monoclonal vimentin antibody (d), or labelled with the polyclonal vimentin antibody (b). Note almost complete disruption of endogenous desmin/vimentin networks in pDDV expressing cells (c, e, h, i). The monoclonal vimentin antibody was much less capable of staining disturbed (dotted) desmin pattern (d). Remarkable was the dotted nuclear desmin staining pattern observed in a large number of cells (e, h, i; white arrows). No clear longitudinal alignment of desmin could be observed in differentiating cells expressing pDDV (h, i). Bar indicates 10 μ m.

complete disruption of endogenous desmin and vimentin filaments was detected (Fig. 2). In control C2C12 cells a normal filamentous desmin and vimentin pattern was detected. In a number of undifferentiated C2C12 cells which expressed the pDDV construct a cytoplasmic desmin staining (filamentous, diffuse or dotted) was observed next to a dotted nuclear desmin staining in close proximity to the nucleus (Fig. 2; arrows) when using a polyclonal desmin antiserum (pDes).

In C2C12 cells induced to differentiate, expression of pDDV did not result in an increase of the dotted desmin staining. The number of cells displaying a more diffuse network increased, although cells with large desmin aggregates next to filaments and dots remained present. Furthermore, dotted nuclear desmin staining was observed in differentiating cells, still showing a completely disturbed desmin/vimentin staining pattern (Fig. 2). In differentiating control C2C12 cells the desmin filaments were oriented in a more or less longitudinal fashion. In the pDDV expressing cells this longitudinal desmin orientation could not be observed.

pVDV-expression in vitro

Transient pVDV expression in C2C12, HeLa cells, and hamster lens cells, caused complete disruption of endogenous vimentin and/or desmin filament networks as determined by indirect immunofluorescence assays using antibodies directed to vimentin and desmin. The mutant desmin and endogenous vimentin and desmin colocalized in fluorescent dots (Fig. 3).

Incubation of the pVDV transfected cells with a monoclonal vimentin antibody (RV202) resulted in a weak staining (Fig. 3b), while use of a polyclonal vimentin antibody (pVim) yielded normal, intense vimentin staining. This suggests that the disruption of vimentin filaments and the concomitant relocation of vimentin rendered the epitope less accessible for the monoclonal vimentin antibody (RV202).

Shortly (6-8 hours) after transfection very small desmin dots were observed, indicative of low level pVDV expression. At this stage complete disruption of endogenous vimentin/desmin networks was already observed, illustrating the dominant-negative effect of pVDV expression. Keratin filaments in HeLa cells remained undisturbed at this stage (not shown). Later after transfection (48 hours) the majority of cells contained large clumps of mutant desmin, indicative of high levels of pVDV expression (Fig. 3). At this stage, not only endogenous vimentin but also the keratin filament network of HeLa cells appeared to be affected by the pVDV overexpression (not shown).

Expression of pDDV and pVDV in transgenic mice

After removal of plasmid sequences both the pDDV and pVDV constructs were introduced into the mouse germ line via pronuclear microinjection. Southern blot analysis of tail DNA of mice born from microinjected zygotes showed that 3 out of 124 had incorporated copies of the pDDV construct and, 9 out of 61 had incorporated copies of the pVDV construct into their genome. Two of the F₀-pDDV mice (strains 9 and 89) produced offspring which inherited the pDDV sequences. Of the nine F₀-pVDV mice (F₀-strains 5, 7, 9, 33, 47, 50 and 55), seven produced offspring which inherited the pVDV construct. Southern blot analysis revealed no detectable rearrangements in both constructs (not shown). Offspring from pDDV-founders 9 and 89 contained ~2 copies, while in offspring from pVDV-founders copy numbers varied from 2 to ~50. All transgenic mice appeared healthy.

Mouse tissues of pDDV- and pVDV-mice were dissected and split in two. One part was used for RNA analysis, the other part was used for immunofluorescence and immunoblotting assays. Polyclonal antibodies to desmin (pDes) and vimentin (pVim) were

Figure 3: Single- and double-label indirect immunofluorescence assay of HeLa cells and hamster lens cells transiently transfected with pVDV. Cells were incubated with polyclonal antisera to desmin (pDes) and vimentin (pVim). a-d) Double-label staining with pDes (a, d) and a monoclonal antibody to vimentin (RV202; b, c) of pVDV transfected HeLa cells, showing a punctate staining pattern. e, f, i, j) Hamster lens cells double-labelled with the polyclonal desmin antiserum (pDes; e, i) and the polyclonal vimentin antiserum (pVim; f, j). Note the complete disruption of vimentin filaments, absence of intact mutant desmin filaments, and colocalization of mutant desmin and vimentin. g, h) Staining with the polyclonal desmin antiserum (pDes) of HeLa cells, expressing pVDV at relatively low (punctate staining) or very high levels (cells filled with mutant desmin). Bar indicates 10 μ m.

Figure 4: Indirect immunofluorescence microscopy of tissue sections derived from pDDV transgenic mouse 43 (offspring of strain 9). Sections were stained with the polyclonal desmin antibody. In skeletal muscle (a, b) a strong diffuse fluorescence staining pattern was observed in some striated muscle cells. In some cells a dotted desmin staining, next to the diffuse reaction was present (b; arrow). In heart sections only very few cells displayed the strong diffuse desmin staining pattern (c; arrow), whereas some cells showed only a weak diffuse desmin reaction (d). The strongest diffuse staining intensity was observed in striated muscle cells of the tongue (e). In esophagus, only a very few cells displayed

used in these latter assays.

pDDV-expression in mouse tissues

Sections of different tissues of F_0 -mice and their offspring expressing pDDV were analyzed for desmin and vimentin expression. Using the immunofluorescence technique,

Figure 5: Indirect immunofluorescence microscopy of tail sections (a-f) and blood cells (g-j) from transgenic pVDV mice, using polyclonal antisera to desmin and vimentin. a-c) Staining of tail sections of mouse strains 47 (a), 55 (b), and 50 (c) with the polyclonal desmin antiserum. Note the strongly fluorescent dots and clumps (not observed in control tissue) in connective tissue. d-f) Tail sections from mouse strains 47 (d, e), and 55 (f), stained with the polyclonal vimentin antiserum. Vimentin expression was detected in virtually all connective tissue (d), while pVDV expression was detected in a minority of connective tissue (compare a-c to d). Identification of connective tissue containing completely disrupted vimentin filaments using the polyclonal vimentin antibody pVim was hampered by the presence of many cells containing intact filaments. However, in areas less densely packed with mesenchymal cells complete vimentin disruption could be observed (e, f). g and h) Blood smears from strains 47 (g) and 55 (h), incubated with the polyclonal desmin antibody pDes. Note strongly fluorescent dots and clumps (not observed in control blood cells) in a small percentage of blood cells. i and j) Blood smears of strains 47 (i) and 55 (j) stained with the polyclonal vimentin antiserum pVim. Note complete disruption of vimentin filaments in blood cells (i; lower panel). Also partial disruption was observed in these cells (i; upper panel). Bars indicate 4 μ m.

Figure 6: Indirect immunofluorescence microscopy of skeletal muscle (a, b) and cross-striated muscle cells of the tongue (c, d) from pDDV transgenic mice 47 (a, c) and 50 (b, d), using the polyclonal desmin antiserum. Note strongly fluorescent, diffuse desmin staining (not observed in non-transgenic mice) in a minority of muscle fibers. The striated pattern normally observed upon desmin staining was also present in these muscle fibers. Bar indicates 10 μ m.

pattern was seen in smooth muscle cells and myoid cells (data not shown).

Using the polyclonal vimentin antibody (pVim) the pDDV expressing, cross-striated muscle cells in tongue, esophagus and heart showed a weak, diffuse staining pattern, in comparison to the desmin staining pattern. In skeletal muscle a relative strong reaction was obtained in cells containing the pDDV-construct. This staining was as strong as the desmin staining described above. In other tissues no abnormal vimentin staining pattern was detected.

pDDV-expression in mouse tissues

Tail sections of a number of offspring of pDDV-mice showed a punctate staining pattern in connective tissue cells (fibroblasts, endothelial cells) with the polyclonal desmin antibody (pDes) (Fig. 5a, b). Surprisingly, pDDV expression could not be detected in all vimentin expressing cells. In the tail tissue sections about 1%-5% of the connective tissue cells were stained with the desmin antibody. The percentage of cells containing detectable levels of mutant desmin varied considerably between litter mates.

Immunostaining of blood cells using the pDes antiserum resulted in a positive staining of leucocytes, where mutant desmin spots and clumps could be observed (Fig. 5g, h).

skeletal muscle and cross-striated muscle of tongue and esophagus showed a divergent staining pattern in a few (<1%) muscle cells, as detected with the polyclonal desmin antibody (pDes). A very

strong, diffuse staining pattern could be observed in these cells (Fig. 4a, b), while in a few cells desmin dots were seen (Fig. 4b: arrow). In the non-affected muscle cells a normal cross-striated staining pattern was detected. In heart muscle of pDDV-mice only occasionally a diffuse staining pattern was observed (Fig. 4c, d; arrow indicates a pDDV positive cell). The staining intensity in affected heart cells was less as compared to the affected cells observed in skeletal muscle. Also in striated muscle cells of the tongue a strong diffuse staining could be detected in $\pm$ 1% of the cells (Fig. 4e). In tissue sections of esophagus, pDDV positive skeletal muscle cells were only occasionally detected (Fig. 4f, arrow). In all pDDV positive cells desmin cross-striation did not seem to be affected by the mutant desmin expression, however, in areas with extremely high mutant desmin expression this could not be determined. No abnormal staining pattern with the polyclonal desmin antibody (pDes) was found in smooth muscle cells, while in tissue sections of kidney, liver, spleen and testis a normal desmin expression

Figure 7: Northern blot analysis of 10 μ g of total RNA isolated from different transgenic pDDV mouse tissues. Blots were probed with desmin (Des), vimentin (Vim), and actin (Act) probes subsequently. Lanes 1 and 2 represent offspring of pDDV strain 9; lane 3 represents offspring of pDDV strain 89; lane C represents a control mouse which did not carry the pDDV construct. pDDV mRNA was only detected in heart, skeletal muscle, cross-striated muscle cells of tongue and esophagus and in testis (for details see text). No mutant transcript was detected in spleen, kidney, ovary, brain, and liver.

Staining with the polyclonal vimentin antibody (pVim) revealed that expression of the truncated desmin caused disruption of the vimentin filaments in these blood cells (Fig. 5i, j).

Also other tissues of pVDDV-mice were sectioned and stained with the polyclonal antibodies to desmin (pDes) and vimentin (pVim). A normal staining pattern was detected with these antibodies in sections of heart, esophagus, kidney, liver, spleen and testis. In contrast, skeletal muscle and the cross-striated muscle cells of tongue, showed diffusely stained cells with the pDes and the pVim antisera (Fig. 6). Expression of mutant desmin was observed in $\pm 1\%$ of the cross-striated muscle cells. No fluorescent dots or clumps could be observed, but instead the staining pattern was diffuse and much more intense than endogenous desmin staining, suggestive of high levels of mutant desmin expression (Fig. 6). Control sections of non-transgenic mice did not display this staining pattern.

Using the pVim antibody the same staining pattern was detected in the mutant desmin containing cells. In connective tissue cells and blood cells without a mutant desmin staining pattern, a filamentous vimentin staining pattern was occasionally observed. No vimentin staining was seen in cross-striated muscle cells without a mutant desmin staining pattern.

The strong diffuse desmin staining reaction in cross-striated muscle cells must be mainly the result of mutant desmin expression and can only to a minor extent caused by endogenous mouse desmin. This conclusion is based on the fact that staining with polyclonal antibodies against vimentin, resulted in the same diffuse, strongly fluorescent staining pattern as seen for pDes (not shown).

The overexpression of pVDDV and the concomitant disruption of the endogenous vimentin filament network in a significant percentage of stromal- and blood cells did not seem to

Figure 8: Northern blot analysis of 5-20 µg of total RNA from transgenic pDDV mouse strains 47, 50, and 55, isolated from heart, skeletal muscle. Blots were subsequently hybridized to a desmin, a vimentin, and an actin probe. The actin probes recognizes both α - (1.8 kbp) and β/γ -actin transcripts (2.1 kbp). Between hybridizations, blots were stripped. Total RNA (1-2 µg) from desmin and vimentin expressing BHK-21 cells and from pDDV expressing fibroblast cell line 55 (F_0 -55) were included as controls. Lanes C) RNA from a non-transgenic control mouse; lanes 1) F_0 -mouse 55; lanes 2) strain F_1 -55; lanes 3) F_1 -strain 47; lanes 4) strain F_1 -50. Note high levels of pDDV expression in skeletal muscle.

genic tissues. The amount of pDDV transcripts present in offspring of pDDV strains 9 and 89 varied between animals derived from these two founders. In offspring of pDDV strain 9, skeletal muscle (amount of pDDV transcript equals endogenous desmin transcript) and tongue (pDDV transcript twice the endogenous desmin level) displayed the highest expression levels. Also in esophagus (pDDV transcript 0.5 times endogenous desmin level) and testis (but not in ovary) mutant transcripts were found. Offspring of pDDV strain 9 contained mutant transcripts in myocard, whereas in offspring of pDDV line 89 this could not be detected (Fig. 7). The levels of transcripts present in heart muscle of offspring of pDDV strain 9 varied between 0.5 to 2 times that of endogenous mRNA (not shown). In general, expression levels in offspring of pDDV strain 89 were lower compared to the expression levels in offspring of pDDV strain 9. No mutant transcripts could be detected in tissue sections of the brain, spleen, kidney and liver. These Northern blotting results clearly demonstrate that pDDV expression is limited to muscle tissue.

cause detectable developmental, morphological or physiological abnormalities.

Levels of pDDV and pVDD mRNA in transgenic mice

Expression of pDDV and pVDD mRNA, and its influence on endogenous desmin and vimentin expression levels, were analyzed by Northern blotting using mRNA of heart, skeletal muscle, esophagus, tongue, spleen, kidney, liver and testis or ovary. Blots were hybridized to desmin, vimentin, and actin specific probes, subsequently. Actin mRNA levels served as standard. After each hybridization, blots were stripped before the next hybridization was performed. In muscle tissue of control mice only the endogenous desmin mRNA was observed. Expression levels were determined by densitometric scanning of autoradiographs from Northern blots containing equal amounts of RNA.

mRNA-levels of pDDV

As compared to non-transgenic mice, in skeletal muscle tissue of the mice transgenic for pDDV (strains F_0 -9 and F_0 -89) an additional band was present next to the desmin mRNA band, corresponding to the correctly sized pDDV transcript (Fig. 7). The levels of endogenous desmin and vimentin mRNA did not differ significantly between control and trans-

Figure 9: Western blot analysis of extracts from tissues of transgenic pDDV mouse 43 (offspring of strain 9). Blots were incubated with the polyclonal desmin antiserum (panel A) and subsequently with the polyclonal vimentin antiserum (panel B). Mutant pDDV protein was only detected in heart, skeletal muscle, tongue and esophagus. Note that the amount of mutant protein is much lower than endogenous desmin protein. Only a low level of vimentin was present in skeletal muscle, as compared to heart, tongue and esophagus. Panel C shows the difference in pDDV-expression between F₁-mice 89 and 9.

Mutant desmin protein levels in transgenic mouse tissues

Western blotting of pDDV tissues

Western blots of protein extracts derived from the transgenic pDDV mouse tissues were incubated with a polyclonal desmin antibody (pDes; Fig. 9) and a polyclonal vimentin antibody (pVim; Fig. 9). Staining with the pDes antibody demonstrated that the pDDV encoded protein could only be detected in cross-striated muscle of heart, skeletal muscle, tongue and esophagus at 40 kDa (Fig. 9A). After staining with the pVim antibody in all tissues the endogenous 57 kDa vimentin could be detected, while with the vimentin antibody only in skeletal muscle, heart, esophagus and tongue the pDDV product could be found (Fig. 9B). No mutant desmin was detected in testis. In all other tissues investigated no pDDV encoded protein could be detected. The amount of pDDV protein observed in offspring of pDDV strain 89 was always lower (at the threshold of detection) than the amount of pDDV protein in tissues of offspring of pDDV strain 9. Furthermore, no pDDV protein was found in heart muscle in offspring of pDDV strain 89 (Fig. 9C, lane 89).

mRNA-levels of pVDV

Since mouse strains 47, 50 and 55 exhibited the highest pVDV expression levels, they were used for a more detailed analysis of the tissue-specificity and levels of transgene expression. Total RNA samples from various tissues, including heart, skeletal muscle, tongue, spleen, liver, kidney and testis or ovary, were analyzed by Northern blotting (Fig. 8). The three independent strains displayed similar expression patterns of the correctly sized (1.7 kbp) pVDV transcript. High levels of pVDV expression, in the same range as endogenous desmin, were detected in skeletal muscle (Fig. 8) and tongue. In contrast, heart pVDV transcript levels were at the threshold of detection (Fig. 8). Some pVDV expression was detected in esophagus and testis.

Clearly, pVDV expression did not follow the endogenous vimentin expression pattern. Skeletal muscle tissue expresses only low levels of vimentin, while in heart tissue much more vimentin is present (Fig. 8).

Figure 10: Western blot analysis of cytoskeletal fractions of tissues derived from pVDV transgenic F₂-mouse strain 47. The blot was incubated with the polyclonal desmin antiserum (panel A) and subsequently with the polyclonal vimentin antiserum (panel B). The endogenous desmin protein (des; 53 kDa) and the pVDV encoded protein (Δdes; 40 kDa) are indicated. Mutant desmin expression was detected in skeletal muscle and tongue tissue.

Western blotting of pVDV tissues

Mouse tissues of transgenic pVDV mice of offspring of F₀-mice 47, 50 and 55 were used for Western blotting. Protein extracts (Fig. 10A, C) of tongue, esophagus, kidney, liver, spleen, testis, ovary (not shown), brain, eye lens (not shown), heart muscle and skeletal muscle were used.

Correctly sized (40 kDa) pVDV encoded protein was detected in skeletal muscle and tongue (Fig. 10A) and at the threshold of detection in eye lens (not shown). The endogenous desmin (53 kDa) was found in tongue, esophagus, kidney, spleen, liver, testis, ovary (not shown), heart and skeletal muscle. No endogenous desmin could be detected in brain and eye lens. With the pVim antibody (Fig. 10B) only in tongue and skeletal muscle the 40 kDa pVDV protein was detected, while in all tissues the endogenous 57 kDa vimentin was observed.

DISCUSSION

As an approach to studying *in vivo* functions of desmin and vimentin, constructs encoding a truncated desmin protein, were introduced into the murine germ line. Transgenic mice expressed the truncated desmin protein in different cell types, depending on the promoter used in the construct. The truncated desmin protein was chosen on basis of its ability to disrupt both endogenous desmin and vimentin IF networks. This effect was first proven in cell culture studies.

Disruption of mouse desmin and vimentin filaments by pDDV and pVDV expression *in vitro*

Interaction of the mutant and wildtype IF subunits resulted in disassembly of preexisting type III IF networks and inhibits *de novo* IF formation [2, 9, 16, 22, 25, 34, 35, 36, 42, 51, 53].

Expression of pDDV resulted in an almost complete disruption of endogenous desmin and vimentin IF structures in undifferentiated C2C12 cells, proving that the protein derived from pDDV has a truly dominant negative effect on type III IF filaments. However, in differentiating cells, the dominant negative effect seemed to be partially compensated by the increase of endogenous desmin expression, although the effect might also be explained by the relative instability of the mutant desmin protein [35]. However, compared to control cells, differentiating pDDV expressing cells showed a disturbance of

desmin/vimentin filament organization. This resulted in diffuse, hazy filamentous next to dotted staining in myotubes. Furthermore, in differentiating cells no clear longitudinal alignment of desmin was observed. These observations demonstrate that expression of pDDV in myotubes interferes with some basic structural processes in these cells, and therefore should also be capable of interfering with desmin organization in muscle of transgenic mice *in vivo*.

Expression of the pVDV construct in hamster lens cells, HeLa cells and undifferentiated C2C12 cells caused a dominant-negative effect on the desmin and vimentin networks. We conclude that pVDV expression caused disruption of vimentin and desmin filaments *in vitro*, while Raats [37; thesis] and Pieper [31; thesis] have shown an enhanced turnover of the endogenous IF subunits in such cases. A remarkable phenomenon was observed in cells expressing pDDV. In a number of cells cytoplasmic desmin staining (filamentous, diffuse or, dotted) was observed next to, what appeared to be a dotted nuclear staining (Fig. 2e, white arrows). Confocal scanning laser microscopy will have to reveal the exact localization of these structures. These nuclear dots were only observed using a polyclonal desmin antibody. In a previous study [12] it was also shown that in cells stably transfected with C-terminally deleted vimentin subunits, dotlike structures were formed in the nucleus, next to filamentous, dotted and diffuse structures in the cytoplasm. C-terminally deleted keratin filaments have also been observed in the nucleus, where they formed filamentous as well as dotted structures [3]. In contrast to previous observations with myogenic cells expressing a comparable desmin protein under control of the RSV promoter [42], differentiation of C2C12 cells expressing pDDV and pVDV, did not result in an increase of dotted desmin staining.

Regulation of pDDV and pVDV expression

It remains to be elucidated why pDDV is only expressed in cross-striated muscle cells and not in smooth muscle cells. It was previously shown that in transgenic mice a vimentin-desmin hybrid gene (pVVim2, [20]) and a complete desmin gene (pVDes, [29]), both under control of the vimentin promoter, were expressed in virtually all vimentin-producing cells. The pVDes expression pattern was slightly more restricted than that of endogenous vimentin and pVVim2 (no pVDes expression in testis Sertoli and Leydig cells; expression in a subset of vimentin positive cells in some tissues [11, 29]), and pVDes expression levels were lower. The expression pattern of pVDV in turn appears to be more restricted than that of pVDes. In both cases, regulatory elements present in one construct may be missing from the other.

Vimentin is not expressed in mature myotubes [19, 23, 28, 46, 50]. The expression of pVDV in myotubes might be caused by the lack of negative control elements from the vimentin gene suppressing expression in myotubes. However, it has been shown previously [23;28] that the vimentin promoter is sufficient for downregulation of vimentin expression during myogenesis. Alternatively, positive control elements stimulating muscle-specific expression may be present in the desmin gene derived part of pVDV. This would imply that regulatory elements located in different parts of the desmin gene are involved in *in vivo* desmin expression, since it has been shown [24, 28] that the 5' flanking region of the desmin gene contains sequences capable of driving high level, muscle-specific expression in cultured myogenic cells.

Disruption of desmin IF in muscle

During myogenesis, the longitudinally oriented IFs undergo phosphorylation-mediated disassembly and are redeployed to a transverse association along the I-Z-I bands [46]. While intact desmin filaments may be dispensable for *in vitro* myogenesis [42], it has been

shown that *in vitro* myoblast fusion is inhibited when disassembly of longitudinal desmin filaments is blocked by injecting desmin phosphorylation site-specific antibodies [45]. The concomitant occurrence of striated and diffuse staining desmin patterns observed in this study may indicate that the mutant desmin is not capable of interacting with endogenous desmin, thereby leaving the process of myogenesis undisturbed. Conformation of this notion requires electron microscopic analysis of mutant desmin expressing muscle fibers.

***In vivo* functions of desmin and vimentin**

It has been demonstrated that desmin and vimentin filaments are dispensable for *in vitro* myogenesis [42]. Similarly, expression of truncated vimentin did not cause detectable abnormalities during early embryogenesis in *Xenopus laevis* [8]. Furthermore, inactivation of both keratin 8 alleles in embryonic stem cells did not inhibit differentiation into yolk sac-like embryoid bodies [4]. However, disruption of keratin filaments in the skin of transgenic mice [9, 15, 51], in *Xenopus laevis* oocytes [47], and in F9 embryonal cells [48] by dominant negative mutation revealed that keratins can have an important function in providing mechanical support to warrant structural integrity of cells and tissues, demonstrating the feasibility of studying IF function by this approach. The important questions that results from this study is why the pDDV and pVDV expressing mice do not display an abnormal phenotype?

The pDDV and pVDV transgenic mice described here expressed mutant desmin in a minority of desmin- and/or vimentin-expressing cells, respectively. If desmin and vimentin function at the level of tissues or organs [19], e.g., by imparting structural integrity to the cell in the context of its tissue, then the cells expressing the truncated desmin may be 'rescued' by non-expressing neighbouring cells. pVDV-expressing cells which do not constitute a solid tissue, such as blood cells, may be incompetent for certain functions, but non-expressing cells could substitute for the defective cells.

The generation of transgenic mice expressing the truncated desmin in all desmin (pDDV) or vimentin (pVDV) containing cells, or inactivation of the mouse desmin or vimentin genes via homologous recombination in embryo stem cells may be necessary to elucidate the functions of both desmin and vimentin IFs.

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SUMMARY/SAMENVATTING

SUMMARY

During myofibrillogenesis *in vivo* as well as *in vitro* different muscle-specific proteins are expressed at different stages of development. In the first part of this thesis (chapters 1 - 4) *in vivo* and *in vitro* myofibrillogenesis and differentiation are studied using immunofluorescence and immunoblotting assays. For *in vivo* developmental studies early postimplantation mouse embryos are used. The expression pattern of desmin and titin is studied in these mouse embryos during different stages of development (8.0 - 9.5 days of development) in the heart-anlage and the myotomes. Surprisingly, titin is found in the heart rudiment at 8.25 days (Theiler stage 12) in a punctate pattern even before desmin is expressed in the cardiomyoblasts. During further development of the heart at 8.5 days (Theiler stage 13) titin filaments are formed and at 9.0 days (Theiler stage 14) titin cross-striation is detected before desmin cross-striation can be observed. At 9.5 days (Theiler stage 14) also desmin cross-striation was seen in the heart muscle cells. One conclusion from this mouse embryo study is that titin is an earlier marker for cross-striated muscle differentiation than desmin.

To further examine the expression patterns of muscle-specific proteins, *in vitro* hamster muscle cells (BHK21/C13; chapter 2) and human skeletal muscle cells (chapter 3) are studied at several stages of differentiation. In undifferentiated BHK-21/C13 cells and human skeletal myoblasts desmin is detected in a filamentous pattern. None of the specific sarcomeric proteins such as titin, nebulin, sarcomeric myosin, or the tropomyosins can be detected in these undifferentiated cells. Both cell types are induced to differentiate by changing the culture medium from a high nutrition medium to a low nutrition medium. The BHK-21/C13 cells then differentiate from a polygonal phenotype to a more elongated form. The human skeletal muscle cells form myotubes during this *in vitro* differentiation process. Early in the differentiation process of both cell types, first the typical punctated titin expression pattern is seen before other sarcomeric proteins can be detected. Later, in elongated BHK-21/C13 cells and in matured skeletal muscle myotubes, titin is observed in a cross-striated pattern, again before other proteins assembled into a striated pattern. Confirmatory results are obtained from immunoblotting assays and by two-dimensional gel electrophoresis studies. The expression of titin during *in vitro* myofibrillogenesis confirms the results found in the *in vivo* model: titin is one of the first myofibrillar proteins expressed in differentiating muscle cells. Furthermore, the sequential stages of assembly of this protein show that titin is of great importance for arranging other sarcomeric proteins.

In chapter 4 the first characterization of cardiotin is described. The distribution pattern of cardiotin, a structural component of the myocard, is compared to that of other sarcomeric constituents and the sarcoplasmic reticulum in several species and tissues. Cardiotin is localized between the myofibrils of the cardiomyocytes, perpendicularly to the typical cross-striations, for example observed with antibodies to titin and desmin. This localization of cardiotin suggests a possible link with the sarcoplasmic reticulum. In immunoblotting assays cardiotin is detected at a molecular weight of approximately 300 kDa and is classified as a giant muscle protein like titin and nebulin. Cardiotin seems to be expressed late in the progress of muscle cell differentiation, in fact only (several months) after birth.

In the second part of this thesis (chapters 5 - 7) the expression patterns of truncated hamster desmin and vimentin in transgenic mice are described. The functional role of desmin and vimentin intermediate filaments in the context of (muscle) tissue differentiation is examined by expression of various intermediate filament gene constructs and the disruption of endogenous desmin and vimentin networks in transgenic mice. The vimentin-desmin hybrid gene constructs pVvim2 (chapter 5) and pVDes (chapter 6) are expressed in all vimentin-containing cells of transgenic mice. This results in hybrid intermediate filament formation, but has no effect on normal

differentiation or development of the mice. The gene constructs encoding a carboxy-terminally deleted mutant desmin subunit driven by the desmin or the vimentin promoter, pDDV and pVDV respectively (chapter 7), also cause no detectable developmental, morphological, and functional abnormalities, although disruption of desmin and vimentin filaments is seen in part of the cells in the tissues. In contrast to the expression levels of constructs pVVim2 and pVDes, the pVDV construct is expressed in a small percentage of vimentin-containing cells, and surprisingly also in a small percentage of cross-striated muscle cells. The pDDV construct is only detected in a small percentage of striated muscle cells but not in smooth muscle cells. In conclusion, several truncated mutant desmin subunits cause disruption of intermediate filament networks in mesenchymal tissues, while no functional, developmental, and morphological abnormalities in the animals are detected.

SAMENVATTING

Tijdens de spierontwikkeling worden, zowel *in vivo* als *in vitro*, verschillende spier-specifieke eiwitten op verschillende momenten van dit ontwikkelingsproces aangeschakeld. In het eerste gedeelte van dit proefschrift (hoofdstukken 1 - 4) worden de spierontwikkeling en spierdifferentiatie *in vivo* en *in vitro*, beschreven aan de hand van de expressie van spierspecifieke componenten met behulp van immunofluorescentie- en immunoblottingexperimenten.

Voor de *in vivo* experimenten zijn muize-embryo's van verschillende leeftijden (8,0 - 9,5 dagen) bestudeerd. Hierbij is gekeken naar het expressiepatroon van met name desmine en titine in het zich ontwikkelende embryonale hart en in de myotomen. Het is opvallend, dat in het rudimentaire hart (dag 8,25) titine in de vorm van kleine aggregaten voorkomt, nog voordat desmine kan worden gedetekteerd. In een later stadium van de ontwikkeling van het embryonale hart (dag 8,5) vormt titine filamenten in de cardiomyocyten en op dag 9,0 wordt voor het eerst titine-dwarsstreping waargenomen. Dwarsstreping van desmine wordt voor het eerst op dag 9,5 in het embryonale muizehart gevonden. In de myotomen wordt een identieke volgorde van expressiepatronen van deze eiwitten waargenomen. Uit deze studie kan gekonkludeerd worden dat in het muize-embryo titine een vroegere marker voor dwarsgestreepte spierceldifferentiatie is dan desmine, hetgeen eerder beschreven is.

In gekweekte hamster spiercellen (BHK21/C13; hoofdstuk 2) en gekweekte humane skeletspiercellen (hoofdstuk 3) is gekeken naar het expressiepatroon van spierspecifieke eiwitten vóór, tijdens en na het induceren van differentiatie. In ongedifferentieerde BHK21/C13 cellen en humane skeletspiercellen wordt desmine waargenomen in een filamenteuze vorm. Er worden geen andere sarcomeer-specifieke eiwitten, zoals titine, nebuline, sarcomeer specifiek myosine of tropomyosines gevonden in deze cellen. Beide celtypen kunnen *in vitro* worden geïnduceerd tot differentiatie door het voedingsrijke kweekmedium waarin deze cellen normaal prolifereren te vervangen door een voedselarm medium. Na deze 'medium-switch', differentiëren de polygonale BHK21/C13 cellen tot een geëlongeerd fenotype. De humane skeletspiercellen fuseren *in vitro* tot myotubes. Tijdens de eerste fase van differentiatie vertonen beide celtypen een gepuncteerd titine patroon, dat eerder dan andere sarcomeer-specifieke eiwitten tot expressie komt. Gedurende het differentiatieproces wordt titine-dwarsstreping waargenomen nog voordat andere spierspecifieke eiwitten in een dergelijk patroon voorkomen. Deze volgorde van aanmaak van verschillende eiwitcomponenten tijdens het differentiatieproces wordt eveneens gevonden met behulp van immunoblotting- en tweedimensionale gelelektroforese technieken. Het expressiepatroon van titine tijdens de spierontwikkeling *in vitro*, is te vergelijken met de resultaten die gevonden worden in het muize-embryo model (hoofdstuk 1): titine is het eerste eiwit dat zijn plaats vindt in de zich vormende sarcomeren en het lijkt van belang te zijn voor de assemblage en rangschikking van andere sarcomeer-specifieke eiwitten, zoals actine, myosine, tropomyosine en desmine.

In hoofdstuk 4 wordt de karakterisatie van cardiotine beschreven, een nieuwe structurele eiwitcomponent in het myocard. De cardiotine-distributie in het hart wordt vergeleken met die van andere dwarsgestreepte spierspecifieke eiwitten en met de structuur van het sarcoplasmatisch reticulum, in zowel verschillende weefsels als in verschillende species. Cardiotine is gelokaliseerd tussen de myofibrillen van de cardiomyocyt, loodrecht op de zo karakteristieke dwarsstreping die wordt gezien met antisera gericht tegen bijvoorbeeld titine of desmine. De lokalisatie van cardiotine wijst op een mogelijk verband met het sarcoplasmatisch reticulum. Cardiotine heeft op een SDS-polyacrylamide gel een molekulgewicht van ongeveer 300 kDa en wordt, evenals titine en nebuline, beschouwd als één van de zeer grote structurele spiereiwitten. Cardiotine komt laat tot expressie, namelijk pas (enkele maanden) na de geboorte.

In het tweede gedeelte van dit proefschrift (hoofdstukken 5-7), wordt het expressie-

patroon van gemuteerd desmine en vimentine in transgene muizen beschreven. Door verschillende gemuteerde desmine-vimentine hybriden transgeen in muizen tot expressie te laten komen vindt een verstoring van endogene desmine en vimentine filamenten plaats. Hierdoor kan de functie van deze filamenten tijdens de ontwikkeling en differentiatie van (spier-)weefsel worden bestudeerd.

Desmine-vimentine konstrukten geplaatst onder controle van de vimentine promotor, komen tot expressie in alle vimentine bevattende cellen. Dergelijke mutante hybriden onder controle van de desmine promotor worden uitsluitend waargenomen in een klein percentage dwarsgestreepte spiercellen en niet in gladde spiercellen. Deze konstrukten, zowel onder controle van de vimentine promotor als de desmine promotor, hadden echter geen waarneembaar (negatief) effect op het ontwikkelings- en differentiatieproces van de muizen. Konkluderend kan gezegd worden, dat door het tot expressie brengen van verscheidene gemuteerde desmine-vimentine hybriden, verstoring van intermediaire filament-netwerken in cellen van mesenchymale origine veroorzaakt kan worden zonder dat in deze cellen of weefsels funktionele of morfologische afwijkingen kunnen worden waargenomen.

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CURRICULUM VITAE

Gert Schaart werd geboren op 22 februari 1960 te Elst (Gld.). Na het behalen van het HAVO-diploma aan het Helder College te Zetten in 1978, volgde hij van 1978 tot 1982 de laboratoriumopleiding HBO-A (medisch) en van 1980 tot 1983 de laboratoriumopleiding HBO-B (chemisch) respectievelijk aan de Opleiding voor Laboratoriumpersoneel Arnhem-Nijmegen (afstudeerrichting: mikroskopische technieken) en aan de Hogere en Middelbare Laboratoriumschool te Oss (afstudeerrichting: biochemie).

Van mei 1983 tot juni 1991 werkte hij als research-analist binnen het Instituut voor Pathologie (hoofd: Prof. Dr. G.P. Vooijs) van het Academisch Ziekenhuis St. Radboud te Nijmegen onder leiding van Dr. F.C.S. Ramaekers.

Sinds juni 1991 is hij aangesteld bij de vakgroep Moleculaire Celbiologie & Genetica (hoofd: Prof. Dr. F.C.S. Ramaekers) van de Rijksuniversiteit Limburg (RL) als onderzoeks-assistent. Hij participeerde in het onderwijs voor studenten van de Faculteiten Geneeskunde en Gezondheidswetenschappen van de RL. Sinds 1989 is hij cursusleider bij het Trainingscentrum Medische Biotechnologie aan de Hogeschool West-Brabant te Etten-Leur.

Met ingang van 1 december 1993 is hij werkzaam op een door de Nederlandse Hartstichting gesubsidieerd onderzoeksproject.

Gert Schaart is sinds september 1993 gehuwd met Helma Kuijpers.

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