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Molecular Changes of Titin in Left Ventricular Dysfunction as a Result of Chronic Hibernation

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J. Ausma, D. Fürst, F. Thoné, B. Shivalkar, W. Flameng, K. Weber, F. Ramaekers and M. Borgers. Molecular Changes of Titin in Left Ventricular Dysfunction as a Result of Chronic Hibernation. *Journal of Molecular and Cellular Cardiology* (1995) 27, 1202–1212. Cardiomyocytes of chronic hibernating myocardium are affected by partial to complete loss of sarcomeres, accumulation of glycogen, adaptations in size and shape of mitochondria, reorganisation of nuclear chromatin and depletion of sarcoplasmic reticulum. The nature of these changes, which from a purely morphologic viewpoint are akin to dedifferentiation, needed further clarification at the molecular level. For this purpose we have studied the expression and reorganization of titin, one of the earliest markers of cardiomyocyte differentiation. By use of monoclonal antibodies, recognizing different epitopes distributed over the whole length of the titin molecule, we were able to detect changes in its molecular organization as a result of chronic hibernation. The epitopes of the titin molecule attached to the Z-disc and those present close to the M-line remained detectable at all stages of hibernation, while epitopes at the A–I junction and parts of the myosin anchoring region of the molecule became masked or were lost. A fragmented or punctuated appearance of the titin staining pattern with antibodies to A–I junction related epitopes is found in cells which we consider to represent a more advanced stage of dedifferentiation. Changes in the distribution of the titin molecule or its molecular environment in hibernating myocardium resemble at least in part changes occurring during muscle cell differentiation, although in reversed order.

KEY WORDS: Human heart failure; Chronic hibernating myocardium; Cardiomyocyte; Titin; Dedifferentiation.

Introduction

The term "hibernating myocardium" was coined by Rahimtoola (1985) to describe a state of left ventricular dysfunction in patients with coronary artery disease in the absence of myocardial infarction. This process is, at least partially, reversible upon restoration of blood flow to the affected segment. The hibernating response of the heart has been considered as an act of self preservation to cope with a reduced myocardial blood flow (Rahimtoola, 1989). The recovery of function is described to be either rather quick (acute hibernation) or slow to very slow (chronic hibernation) (Rahimtoola, 1989; Ross, 1991; Schelbert, 1991).

Recently Borgers *et al.* (1993a,b) described the morphologic changes of cardiac biopsies obtained from 98 patients with left ventricular ischemic dys-

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function. The typical structural changes, noted in cardiomyocytes of almost all patients, included loss of sarcomeres, the presence of abundant plaques of glycogen, loss of sarcoplasmic reticulum, occurrence of rough endoplasmic reticulum, shape changes of mitochondria and redistribution of nuclear chromatin. It was proposed that segments in which these structural changes prevail are characteristic of chronic hibernating myocardium and most probably are those that do not recover immediately after revascularization. Instead they might show a delayed recovery of function (weeks to months), because structural remodeling requires time in order to regain sufficient contractile material (Rahimtoola, 1989; Schelbert, 1991; Vanoverschelde *et al.*, 1993). The characteristic change of hibernating cells concerns the replacement of contractile material (sarcomeres) by glycogen, a phenomenon that always starts in the perinuclear area of the cell and gradually extends towards the periphery in most altered cells. Since the lack of contractile filaments, the presence of accumulated glycogen (Manasek, 1986) and the preference of glucose over fatty acids as energy source (Harary, 1979) are characteristic features of embryonic cells, these criteria are considered as hallmarks suggesting that hibernating cells are going through a process of dedifferentiation.

In biopsies derived from patients with chronic hibernating myocardium the expression, organization and assembly of markers of cardiac cell development were studied to support the dedifferentiation hypothesis of hibernating cells. Titin was chosen as a dedifferentiation marker. Titin is a giant elastic protein of half sarcomere length, spanning the distance from the Z- to M-line (Fürst *et al.*, 1988; 1989a). Protein sequences derived from cDNA cloning show that the carboxyterminal end of titin is at the M-band (Labeit *et al.*, 1992). It is one of the earliest markers of cardiomyocyte differentiation (Tokuyasu and Maher, 1987a; Wang *et al.*, 1988a; Schaart *et al.*, 1989; Schultheiss *et al.*, 1990; Van der Loop *et al.*, 1992). In this study the sequence of organizational and redistributional changes of titin in hibernating myocardial cells, were monitored with antibodies to different epitopes of the titin molecule.

Materials and Methods

Patients

The human cardiac tissue material used in this study consisted of transmural biopsies obtained from 17 patients at the time of coronary bypass surgery. All patients gave their informed consent. The study was approved by the local ethical committees for research. The detailed individual patient characteristics are described in previous papers (Vanoverschelde *et al.*, 1993; Maes *et al.*, 1994). In brief, all patients had severe LAD stenosis and marked anterior wall abnormalities as evaluated by angiography and 2D echocardiography. The viability of the myocardium was verified by Positron Emission Tomography (PET) and by the assessment of function 3–6 months after coronary bypass surgery. All patient material dealt with in this paper fulfilled the criteria of chronic hibernation as defined previously (Vanoverschelde *et al.*, 1993; Maes *et al.*, 1994).

Morphologic evaluation

Of all patients a first biopsy was fixed for a minimum of 2 h in 3% glutaraldehyde buffered with 90 mM KH_2PO_4, washed in the buffer and postfixed for 1 h in 2% OsO_4 buffered with 50 mM veronal acetate, dehydrated in a graded series of ethanol and embedded in epoxy resin (Epon) (Flameng *et al.*, 1984). Light microscopic evaluation of morphologic changes was performed on 2 μm thick sections of Epon-embedded biopsies, which were stained with periodic acid Schiff (PAS) and 0.1% toluidine blue to quantify the glycogen content and the loss of myofibrils. The degree of cellular change was evaluated in cells where the nucleus was visible in the plane of the section. The number of cells affected by myolysis was obtained by evaluating at least 200 cells per biopsy.

Left ventricle biopsies derived from seven donor hearts, which were either used for orthotopic transplantation or homograft prelevation, also used in a previously published study (Borgers *et al.*, 1993b), were treated as above and served as non-ischemic controls.

Indirect immunofluorescence assays

A second biopsy of the hibernating myocardium was quickly frozen in isopentane pre-cooled with liquid nitrogen. Thick sections (5μm) were air-dried before use, and treated with 0.5% Triton X-100 (BDH Chemicals Ltd., Poole, UK) in phosphate-buffered saline (PBS) for 5 min at room temperature, followed by washing in PBS. The sections were incubated with the primary antibodies for 45 min at room temperature and washed with PBS (three steps of 10 min each). They were subsequently incubated with the secondary, fluorescein isothiocyanate (FITC) conjugated goat-anti-mouse Ig-subclass specific antibody [Southern Biotechnology

Associates (SBA) Inc., Birmingham, USA], or goat-anti-rabbit antibody (SBA) for polyclonal antisera for 45 min and then washed in PBS (three steps of 10 min each).

In the double-labeling procedure the immunostaining steps were repeated with a second primary antibody of another Ig-subclass, the sections were washed in PBS and then incubated for 45 min with the secondary, Texas Red conjugated Ig-subclass specific antibody (SBA, Birmingham, AL, USA).

After these immunohistochemical procedures the sections were placed in distilled water for 5 min, followed by post-fixation in methanol for 5 min. The sections were air-dried and mounted in Mowiol (Hoechst, Frankfurt a.M., Germany) (Osborn and Weber, 1982). Nuclei were routinely stained with 4'-6-diamidine 2-o-phenylindole (DAPI: Sigma Chemicals, St Louis, MO, USA) in a dilution of 1: 10 000 with PBS. As a control, application of the first antibody was omitted.

Confocal scanning laser microscopy

Double label immunostained myocardium sections were observed with a Bio-Rad MRC-600 confocal scanning laser miscroscope (Bio-Rad Laboratories, Richmond, CA, USA) equipped with a Krypton/ Argon mixed gas laser (Ion Laser Technology, Salt Lake City, UT, USA) with two separate wavelengths for the excitation of fluorescein isothiocyanate (488 nm) and Texas Red (568 nm) and mounted on a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Antibodies

The following antibodies were used in this study the epitopes of the titin antibodies have been schematically presented in Fig. 1): (1) pTitin, a rabbit polyclonal antibody against titin which reacts with the A band and the A–I junction but not with the I-band or the Z-disc (Gassner, 1986). (2) A mouse monoclonal antibody 9D10 to titin. This antibody recognizes an epitope in the I-band at the A–I junction in skeletal and cardiac muscle (Wang and Greaser, 1985; Wang *et al.*, 1988a; Greaser *et al.*, 1989; Handel *et al.*, 1989). The 9D10 monoclonal antibody was developed by M. Greaser and obtained from the Developmental Studies Hybridoma Bank maintained by the department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore MD 21205, USA and the department of Biology, University of Iowa, Iowa City IA, USA, under contract N01-HD-2-3114 from the NICHD. (3) T30, a mouse monoclonal titin antibody which reacts with repetitive sequences of the A-band. T30 detects five of the seven C-stripes, that coincide with binding places for myosin associated proteins, i.e. the C-protein and H-protein (Fürst *et al.*, 1989a). (4) T31, a mouse monoclonal titin antibody which reacts with repetitive sequences of the A-band. T31 detects both P-stripes that coincide with binding places of the H-protein (Fürst *et al.*, 1989a). (5) T12, a mouse monoclonal titin antibody which reacts with an epitope in the I-band just before the Z-disc (Fürst *et al.*, 1988). (6) SR-1, a mouse monoclonal antibody recognizing striated muscle α-actin (Skalli *et al.*, 1988) (DAKO A/S, Glostrup, Denmark). (7) The mouse monoclonal desmin antibodies DE-R-11 (Debus *et al.*, 1983) (DAKO A/S, Glostrup, Denmark) and RD301 (Schaart *et al.*, 1989; Raats *et al.*, 1991). (8) MF 20, a mouse monoclonal antibody recognizing all forms of myofibrillar myosin heavy chain (Bader *et al.*, 1982). The MF 20 monoclonal antibody was developed by D. Fischman and obtained from the Developmental Studies Hybridoma Bank maintained by the department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore MD 21205, USA and the department of Biology, University of Iowa, Iowa City IA, USA, under contract N01-HD-2-3114 from the NICHD. (9) MF 30, a mouse monoclonal antibody, it recognizes the S2 fragment of the myosin heavy chain of various neonatal and adult species (Bader *et al.*, 1982). The MF 30 monoclonal antibody was developed by D. Fuschman and obtained from the Developmental Studies Hybridoma Bank maintained by the department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore MD 21205, USA and the department of Biology, University of Iowa, Iowa City IA, USA, under contract N01-HD-2-3114 from the NICHD. (10) The mouse monoclonal antibody TM311 which reacts with tropomyosin (Sigma Immunochemicals, St. Louis, USA). (11) The mouse monoclonal vimentin antibody RV203 (Schaart *et al.*, 1991). In addition rhodamine-labeled phalloidin was used to stain F-actin (dilution 1:80 in PBS) (Molecular Probes Inc. Eugene, OR, USA).

Results

Morphological changes

The most important change in cellular substructure seen in a considerable number of myocardial cells

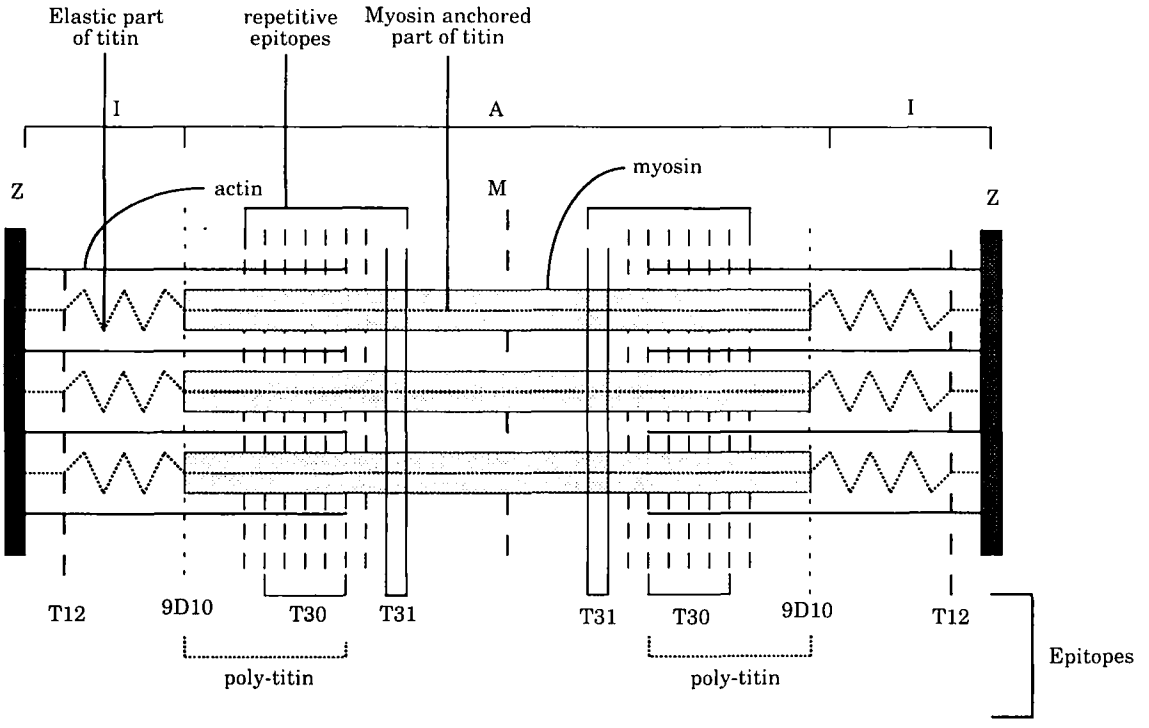

Figure 1 Schematic representation of the topography of the epitopes recognized by the different anti-titin antibodies. Z: Z line, M: center of the M-band. A and I mark the anisotropic and isotropic bands respectively.

from chronic hibernating myocardial segments was the depletion of contractile material without loss of cell volume. Loss of contractile material was in many cells limited to the vicinity of the nucleus, but in others it comprised the bulk of the cytoplasm, leaving only a few or no sarcomeres at the periphery of the cell (Fig. 2a). The myolytic areas, being the spaces in which myofilaments were dissolved, became occupied by glycogen and many small mitochondria (Fig. 2b). Cells were considered as affected by the process of myolysis when more than 10% of the cell volume was occupied by glycogen (Borgers *et al.*, 1993b). Within a group of 17 patients $25 \pm 15\%$ of all cells were affected. An increase in connective tissue was consistently observed in areas in which structurally affected myocardial cells prevailed (Fig. 2a).

Immunofluorescence assays

In parts of the myocardium which contained only normally structured cells the antibodies pTitin, T30 and 9D10, which label three different epitopes in the A–I junction part of titin (Fig. 1), showed a similar double-banded cross-striated pattern (Fig. 3a). Hibernating cells with perinuclear myolysis showed the same distribution pattern. However, in those cells the cross-striated staining pattern was limited to the peripherally located sarcomere strands (Fig. 3b). In most hibernating cells with a severe degree of myolysis, the double- banded staining pattern was absent for the three antibodies. This disappearance of a titin cross-striated staining pattern for the antibodies 9D10, T30 and PTitin seems to be a stepwise process, the earliest observed change was the loss of double-banded titin staining and the occurrence of the titin staining pattern as single bands (Fig. 3c). In addition, the staining intensity was markedly lower as compared to normal cells. In more severely affected cells the striation patterns of titin normally crossing the whole cardiomyocyte, was limited to certain parts of the cell. The titin striations were lost, only locally some short titin striations remained (Fig. 3c). In the most severely affected areas, 9D10, T30 and pTitin showed an even more distinct decrease of the titin cross-striations. Many cells lost their cross-striated staining pattern or showed a punctate (dot-like) staining reaction (Fig. 3d).

In contrast to pTitin, 9D10 and T30 the cross-striated staining pattern of T12, which recognizes an epitope at the I-band just before the Z-line, and T31 recognizing two repetitive epitopes close to the centre of the A band, were retained throughout the

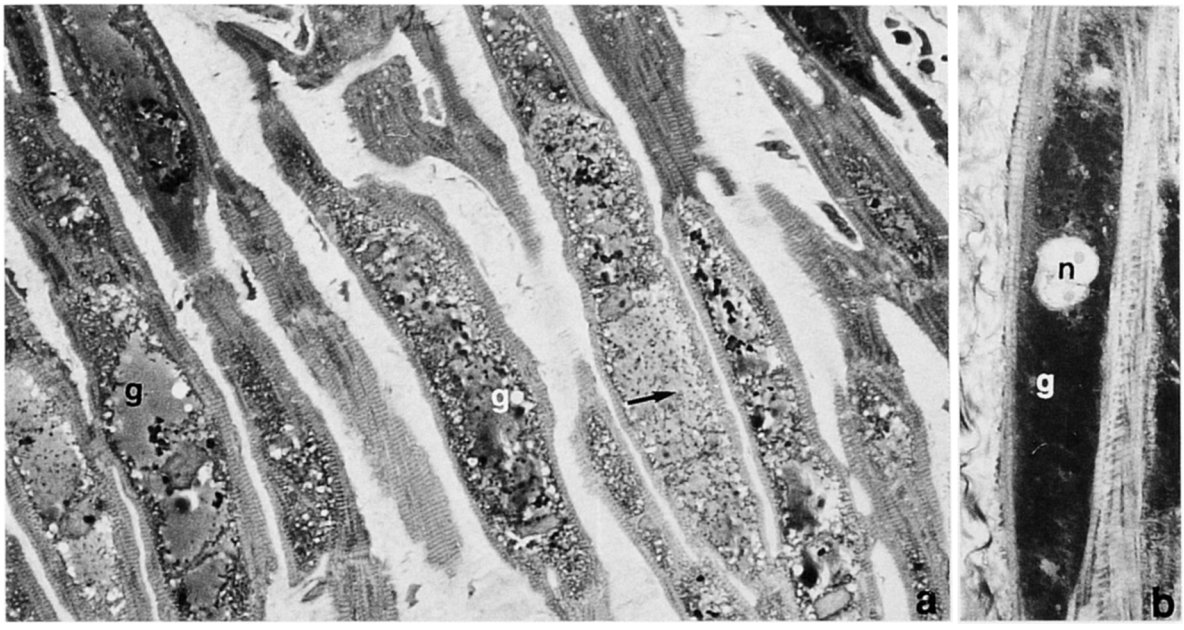

Figure 2 Light microscopy of morphologic changes in chronic hibernating myocardium. (a) Two micron thick section of an affected area, stained with toluidine blue showing that the centers of most cells are myolytic. The myolytic areas are filled with amorphous material (glycogen, g) intermingled with small dots (mitochondria, arrow). Increased amounts of connective tissue material surround the cardiomytes. Magnification: × 540. (b) Severely hibernating cells with large amounts of glycogen (g) seen as dark zones surrounding the nucleus (n) subsequent to PAS staining. Magnification: × 700.

various stages of myolysis (Fig. 4a–d). With these two antibodies titin striations were seen at a regular distance in the sarcomeres of the hibernating cells. There were no differences in the staining patterns with these anti-titin antibodies in different zones of the chronic hibernating myocardium (Fig. 4a,c). From double-labeling immunofluorescence studies it was obvious that the organization of titin nearby the Z-line (T12) and close to the centre of the A-band (T31) was still intact while in the same cells the cross-striated titin staining patterns for epitopes at the first part of the A-band and A–I junction were completely lost (Fig. 4a,b and 4c,d).

In contrast to the titin disorganization, other sarcomeric proteins remained well organized in the sarcomeres. Even in zones with severe changes in 9D10, T30 and pTitin staining, actin, myosin, tropomyosin and desmin still showed clear cross-striations, similar to those as seen in the sarcomeres of normal myocardium. As an example, the staining pattern of desmin in relation to the titin 9D10 epitope is shown in Figure 5a and 5b. In Figure 5c and 5d confocal laser scanning microscopic pictures of myosin and titin are shown. The sarcomeres in this figure have a clear cross-striated myosin pattern, while the titin epitope recognized by 9D10 is scarcely visible. α-smooth muscle actin became re-expressed in chronic hibernating myocardium cells, but vimentin expression was not detected in the dedifferentiating cardiomyocytes.

In order to investigate the localization in relation to the Z- and M-line and distance of different titin epitopes during stages of dedifferentiation of chronic hibernating myocardium confocal scanning laser microscopy analysis was performed. The titin epitopes recognized by T31 never coincide with the Z-disc (desmin positive), indicating that the titin epitopes at the centre of the A-band have not dramatically changed their position within the remaining sarcomeric structure.

Discussion

Cardiomyocytes of chronic hibernating myocardium undergo typical ulstrastructural changes of which the replacement of sarcomeres by glycogen is the hallmark (Borgers *et al.*, 1993b; Ausma *et al.*, 1994). These observations have been considered as morphologic indicators of cardiomyocyte dedifferentiation. In addition, recent studies (Ausma *et al.*, 1995) showed that α-smooth muscle actin, a protein that gradually disappears from cardiomyocytes during development, became re-expressed in hibernating cardiomyocytes. Also titin, which is one of the earliest sarcomeric proteins

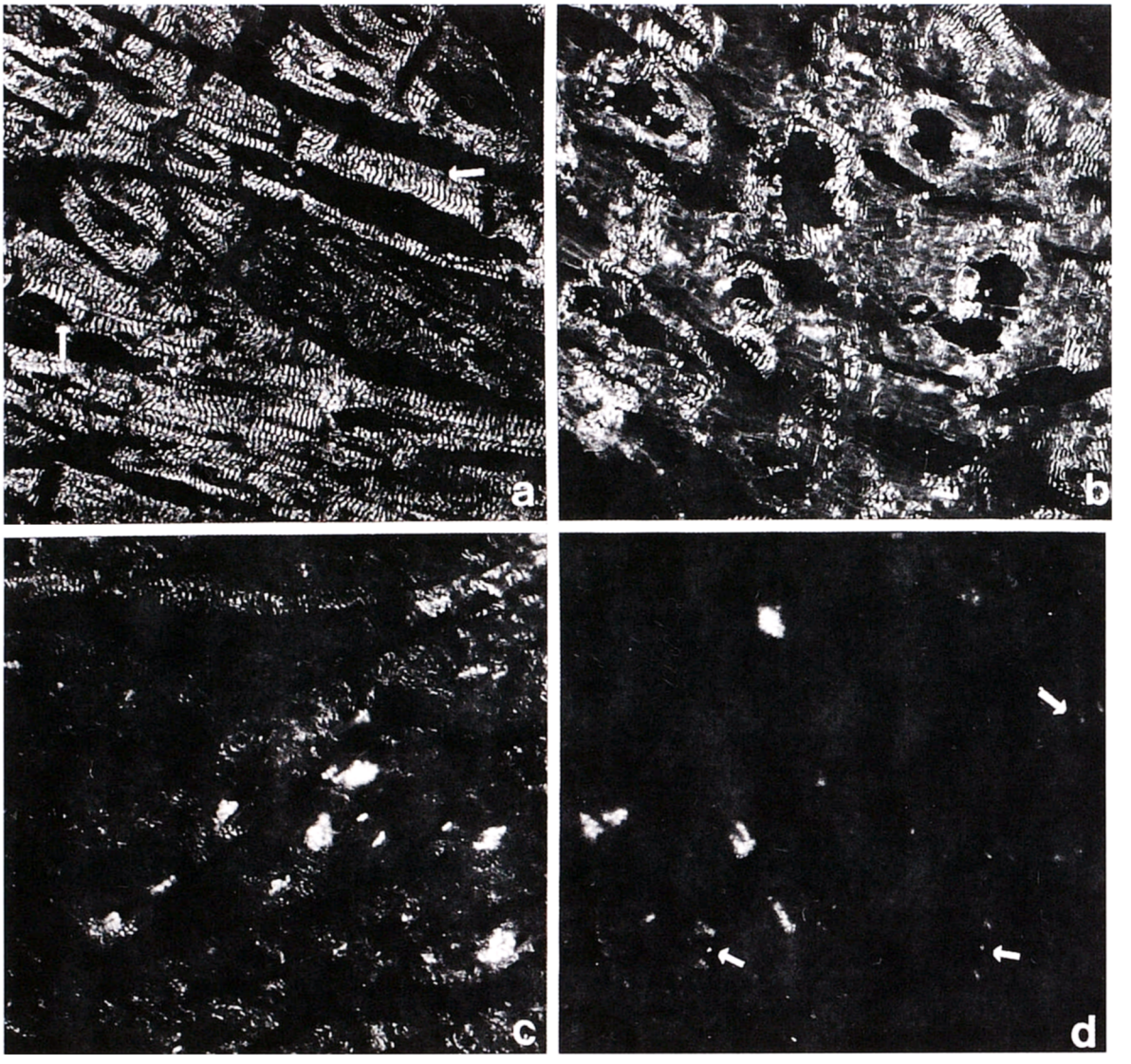

Figure 3 Immunofluorescence micrographs of frozen sections of myocardium of patients with chronic hibernation, incubated with antibodies against the A–I junction part of titin. As an example the 9D10 staining is shown. (a) Titin staining in a nearly normal pattern. Double-banded cross-striations are indicated by arrows. (b) Cells in which sarcomeres are located only at the periphery of the cell. Most of these cells showed typical double-banded striations at their periphery. (c) Marked decrease of titin staining in chronic hibernating myocardium: only short titin striations and titin punctations. (d) Only a residual staining is present (arrows). The large dots which are visible around the nuclei represent lipofuchsin autofluorescence. Magnification: × 500.

during embryogenesis, appeared to change its immunocutochemical detectability in hibernating cardiomyocytes. Other sarcomeric and cytoskeletal proteins such as actin, myosin, tropomyosin and desmin remained intact in sarcomeres at the periphery of the hibernating cardiomyocytes.

Effects of hibernation on the titin molecular structure

Studying the organization of titin with monoclonal antibodies against different epitopes situated along this large molecule provides new insights into structural changes of this protein during hibernation. The use of antibodies directed to the epitopes of the titin molecule present at the A-band and the A-I junction of the sarcomere revealed that this part of the molecule is masked or lost in hibernating cells. On the other hand, the epitope of T12, localized in close proximity to the Z-line and the T31 epitope, known to be situated at the centre of the A-band, remained detectable during the process of heart muscle cell adaptation. These results strongly indicate a change in the structure of the titin molecules, in particular, in its normally rigid middle

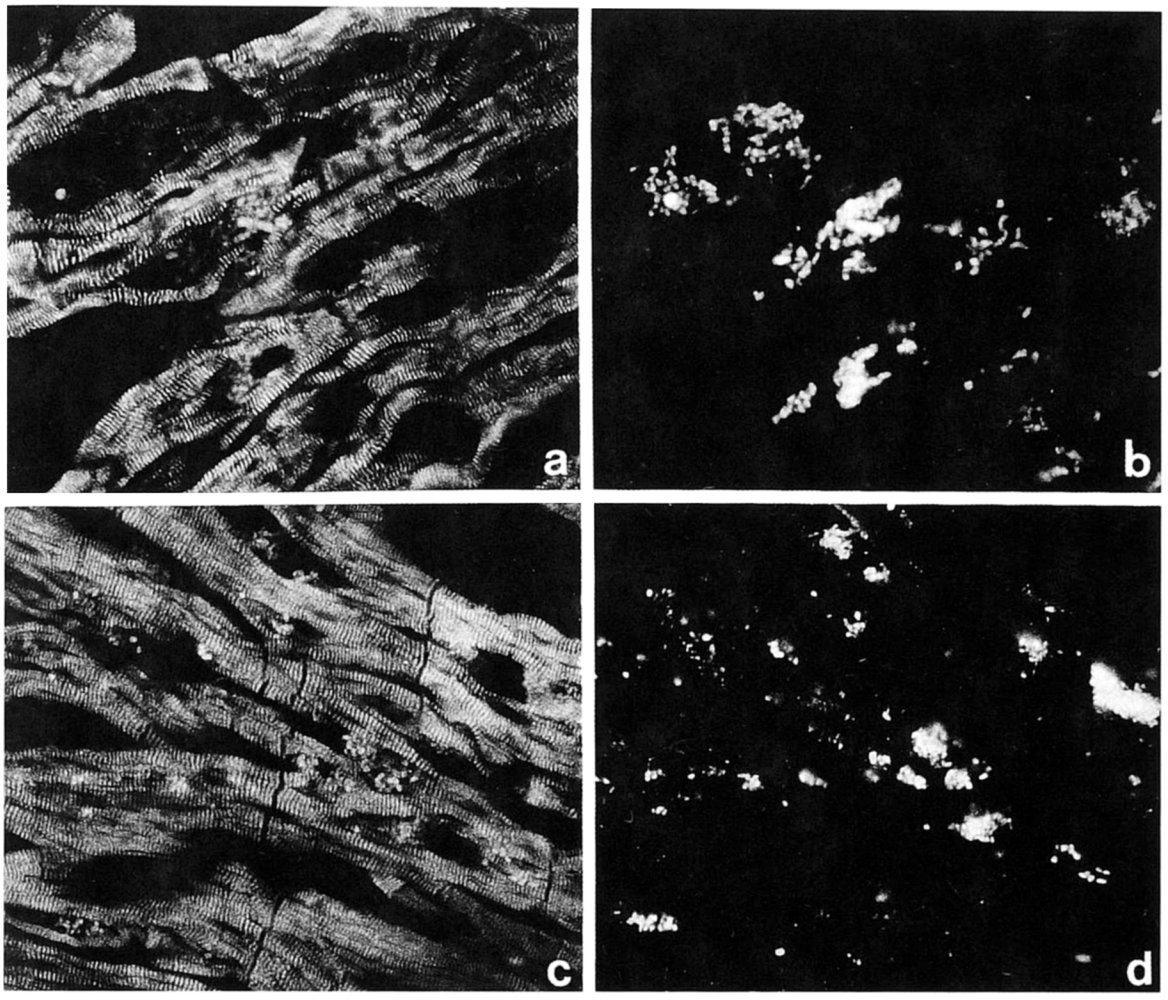

Figure 4 (a,b) Double-labeling of a section of chronic hibernating myocardium with two anti-titin antibodies, one which recognizes the I-band part of titin nearby the Z-line (T12) and the other recognizes the A–I junction part (9D10). Note that T12 staining remained intact in severely affected cardiomyocytes (a), while 9D10 staining was almost completely lost (b). (c,d) Double-labeling of a section of chronic hibernating myocardium with two anti-titin antibodies, one which recognizes the A–I junction part (9D10) and the other which recognizes the A-band part of titin nearby the M-line (T31). T31 staining remained intact in the severely affected cardiomyocytes (c), while 9D10 staining was totally absent (d).

part. In the N-terminus (Z-line part) and the C-terminus (M-line part) of titin seems to remain intact during hibernation. Changes at the molecular level, either occurring in the titin molecule itself or in its molecular assembly with other sarcomeric proteins, apparently start at the part of titin anchored to myosin (recognized by pTitin and T30) as well as the junction between the elastic and rigid part of the molecule (recognized by 9D10). The absence of titin staining at this part of the molecule can be the result of proteolytic degradation, but it is also possible that it results from a change in the molecular environment of the titin molecules. When analysed in more detail, T30 and T31 monoclonal antibodies were shown to recognize repetitive epitopes in the titin molecules, that coincide with the binding places of specific myosin associated proteins, i.e. C-protein and H-protein. The T31 epitope matches with the two P-stripes, which contain only the H-protein. The T30 epitope matches with five of the seven C-stripes, places on which both C-protein and H-protein are attached to myosin. It has been suggested that C-protein interacts with titin (Fürst *et al.*, 1992, Koretz *et al.*, 1993). Differences in titin epitope detectability during (de)differentiation might well be related to changes in the molecular environment of titin, resulting from a change in the interaction between C-protein and titin.

The A-band region of titin has been shown to become extensible when it is detached from myosin (Wang *et al.*, 1988b; Higuchi *et al.*, 1992). Higuchi *et al.* (1992) also showed that during partial depolymerization of thick filaments, the anti-titin anti-

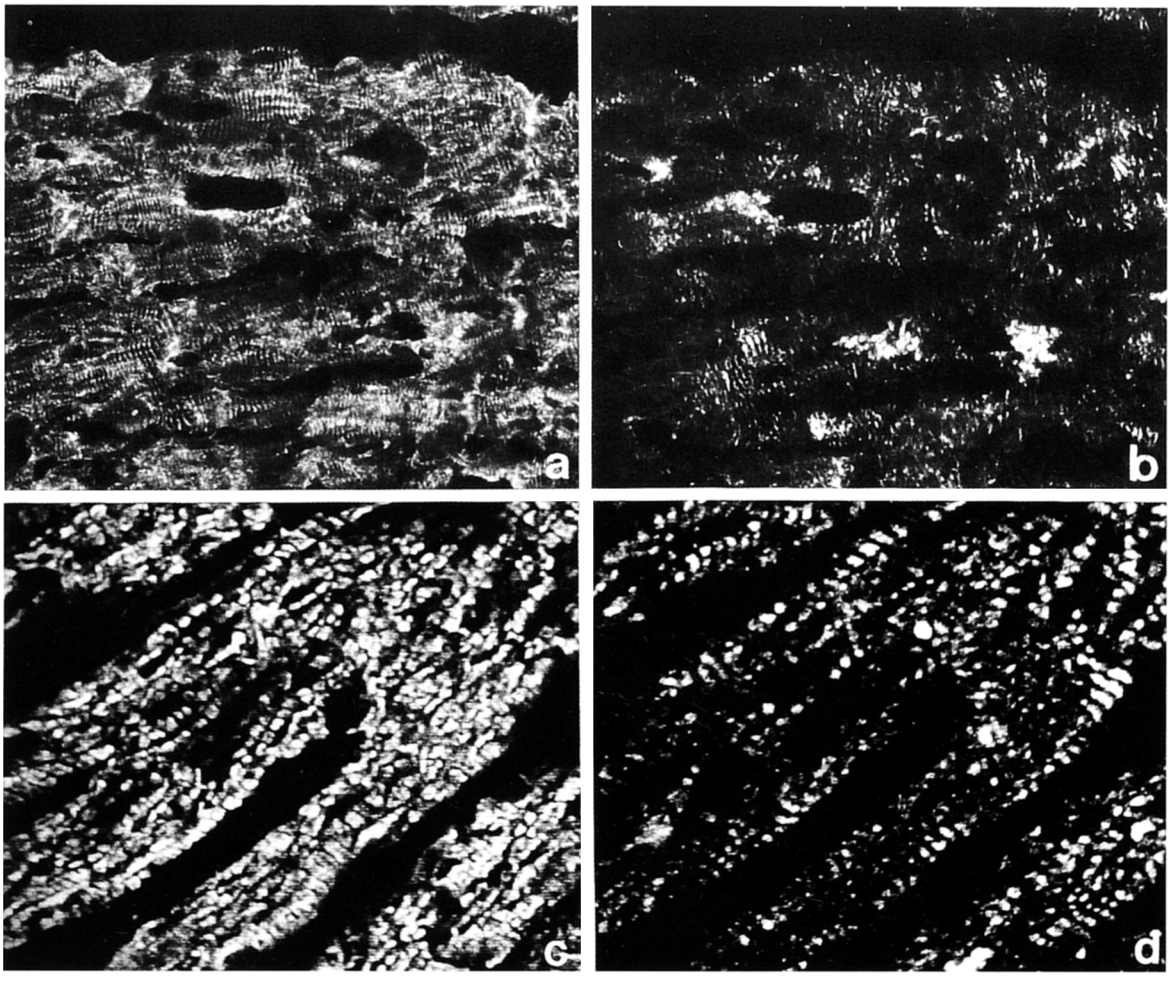

Figure 5 (a,b) Double-labeling of a section of chronic hibernating myocardium with desmin and titin (9D10). (a) Desmin staining still remains intact in these cells while (b) titin, on the other hand is only seen as spots and short striations. (c,d) Confocal laser scanning microscopy of a chronic hibernating segment with myosin staining (MF-20) display in a clear cross-striated pattern (c), whereas only some titin (9D10) is seen (d). Magnification: a,b × 500; c,d. × 1360.

bodies recognizing a specific position in the A-band showed immunoreactivity at a constant distance from the M-line. As the thick filament was almost completely depolymerized, these titin antibodies were found to localize at the Z-line, suggesting detachment of the titin molecule from the M-line anchoring. In chronic hibernating myocardium on the other hand, the thick filaments are still present and as a result T31 immunostaining is still normal, indicating that titin is not detached from the M-line of the thick filaments.

Changes in titin during chronic hibernation: differentiation in reverse?

Borisov (1991) has described that cardiac muscle cells at early, intermediate and terminal stages of differentiation are capable of adaptive remodeling of their contractile system both *in vivo* and *in vitro*. This implies that dedifferentiated cardiomyocytes can redifferentiate again. Moreover, Sharp *et al.* (1993) established that in verapamil-arrested cultured neonatal rat heart cells, in which initially a steep reduction in the amount of actin was observed, the process could be entirely reversed upon omission of the drug. During chronic hibernation hypo/akinesia may lead to a similar reduction in the amount of contractile material, hence indicating a process of dedifferentiation to a state comparable with neonatal cardiomyocytes.

The loss of the titin epitope stained by antibody 9D10 in chronic hibernating myocardial cells started with the fragmentation of titin striations, ultimately leading to dot-like staining. A similar punctate pattern was also seen with 9D10 during cardiac and skeletal myofibrillogenesis at the onset

of the formation of myofibrils (Tokuyasu and Mayer, 1987b; Wang *et al.*, 1988a,b; Schaart *et al.*, 1989; Schultheiss *et al.*, 1990; Van der Loop *et al.*, 1992), and during skeletal muscle cell differentiation in culture (Van der Ven *et al.*, 1992; 1993).

The persistence of a "normal" T31 epitope staining pattern in affected cardiomyocytes seems in conflict with the idea that titin organization starts at the Z-line and progresses towards the M-band (Fürst *et al.*, 1989b) as deduced from comparing changing titin organization during *in vivo* or *in vitro* differentiation processes with that of apparently dedifferentiating hibernating cells. During myofibril formation in mouse skeletal muscle the T30 and T31 epitopes of titin are revealed simultaneously (Fürst, unpublished observation). However, during dedifferentiation in hibernating myocardium, the T31 epitope is still detectable, whereas the T30 epitope is not. It is assumed that the adaptive dedifferentiation process during hibernation takes considerably more time than embryonic differentiation. Therefore, it is possible that such subtle differences in the organization of distinct A-band titin epitopes cannot be detected during skeletal muscle development of the mouse, because of the relatively high speed of differentiation. Likewise, the localization of titin A-band epitopes has not yet been documented in developing human cardiac muscle. Therefore, it cannot be excluded that species differences as well as variations in the organization of the sarcomeres of skeletal and cardiac muscle cause this apparent discrepancy. Also, dedifferentiation of chronic hibernating myocardium is not complete, as indicated by the lack of vimentin expression. The most plausible explanation for the observed differences in titin organization during hibernation and myofibrillogenesis, respectively, may be that the dedifferentiation process is not completely reversible at all the steps of sarcomere assembly.

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