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

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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-
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 redistributational 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 KH2PO4, washed in the buffer and postfixed for 1 h in 2% OsO4 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 (5 µm) 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 immu-
ostaining 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 spe-
cific 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 microscope (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, Ober-
kochen, Germany).

Antibodies

The following antibodies were used in this study the
epitopes of the titin antibodies have been schem-
atically 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 junc-
tion 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 de-
smin 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 my-
ofibrillar 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 de-
partment of Pharmacology and Molecular Sciences,
Johns Hopkins University School of Medicine, Balt-
timore 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 re-
cognizes the S2 fragment of the myosin heavy chain
of various neonatal and adult species (Bader et al.,
1982). The MF 30 monoclonal antibody was de-
veloped by D. Fischman and obtained from the
Developmental Studies Hybridoma Bank maintained
by the department of Pharmacology and Molecular
Sciences, John Hopkins University School of Medi-
cine, 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 vi-
mimentin 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
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 ± 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
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 crossstriations, 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 ultrastructural 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
during embryogenesis, appeared to change its immunocutcheonal 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 center of the A-band, remained detectable during the process of heart muscle cell adaption. These results strongly indicate a change in the structure of the titin molecules, in particular, in its normally rigid middle
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-
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

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 x 500; c.d. x 1360.
of the formation of myofibrils (Tokuyasu and Mayer, 1987b; Wang et al., 1988a,b; Schaart et al., 1989; Schultheiss et al., 1990; Van der Ven 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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References


