Nuclear Lamin Expression in Chronic Hibernating Myocardium in Man

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J. AUSMA, G. J. J. M. VAN EYS, J. L. V. BROERS, F. THONÉ, W. FLAMENG, F. C. S. RAMAEKERS AND M. BORGERS. Nuclear Lamin Expression in Chronic Hibernating Myocardium in Man. Journal of Molecular and Cellular Cardiology (1996) 28, 1297–1305. Cardiomyocytes of chronic hibernating myocardium are known to undergo structural changes, indicative of dedifferentiation. Amongst these are changes in nuclear shape and chromatin distribution. Nuclear A-type lamins are known to be expressed in a differentiation-related fashion and to contribute to nuclear integrity and chromatin organization. Lamin expression was investigated with immunocytochemical staining procedures in biopsies from patients with chronic hibernating myocardium. The expression of A-type (lamin A and C) were shown to be downregulated during hibernation, while lamin B2 remained present in hibernating cardiomyocytes in a way similar to embryonic muscle cells. All heart muscle cells were shown to be negative for lamin B1. The absence of A-type lamins in chronic hibernating cardiomyocytes could be taken as an additional argument for the dedifferentiation state of these cells. The absence of A-type lamins was accompanied by dispersion of the nuclear heterochromatin, in a way similar to nuclei of embryonic cardiomyocytes.

Keywords: Heart failure; Chronic ischemia; Dedifferentiation; Viable myocardium.

Introduction

In recent studies, we described the structural adaptations in cardiomyocytes from patients with chronic hibernating myocardium. The affected segments showed cardiomyocytes with loss of sarcomeres, sarcoplasmic reticulum and T tubules, and presented abundant glycogen plaques, strands of rough endoplasmic reticulum and numerous mini-mitochondria (Vanoverschelde et al., 1993; Borgers et al., 1993; Maes et al., 1994; Ausma et al., 1995a, b). These cellular changes were not considered as degenerative but were interpreted as cellular dedifferentiation. This assumption was supported by the occurrence of “early development” markers of the heart muscle, such as the re-expression of α-smooth muscle actin, the staining of titin in an embryonic-like punctate pattern and the disappearance of cardiokinin, a late marker of the heart muscle development (Schaart et al., 1993; Ausma et al., 1995b). The above mentioned structural alterations were accompanied by particular changes in nuclear shape and composition. The nuclei of chronic hibernating cardiomyocytes were often enlarged and their heterochromatin material was uniformly dispersed, a feature akin to nuclei of embryonic cardiomyocytes (Borgers et al., 1993). These changes led us to investigate the possible involvement of lamins in the hibernation process.

The nuclear lamina is considered to be important for nuclear envelope integrity and the organization of interphase chromatin. It consists of a meshwork of intermediate filament type proteins. Nuclear lamins, subdivided into the A-type and B-type lamins,
are members of a multigene family of proteins that are expressed in differentiation stage-dependent fashion (for reviews see Krohne and Benavente, 1986; Nigg, 1992; Moir and Goldman, 1993). Both A- and B-type lamins may have a role in the expression of genes by directly or indirectly interacting with heterochromatin (Burke and Gerace, 1986; Luderus et al., 1993). B-type lamins (lamins B1 and B2) associate with the inner nuclear membrane during both interphase and mitosis (Gerace et al., 1984). The expression of B-type lamins is found in most vertebrate cell types. In mammals, two A-type lamins have been described: lamins A and C. These two proteins arise from the same gene by alternative splicing (Lin and Worman, 1993).

The expression of A-type lamins is related to a relatively high degree of cellular differentiation. Although, numerous studies on the distribution of lamin subtypes were carried out in developing vertebrate embryos (Lehner et al., 1987; Stewart and Burke, 1987; Röber et al., 1989) and in proliferating cells such as teratocarcinoma cells, hematopoietic cells and a diversity of normal adult tissues (Lebel et al., 1987; Stewart and Burke, 1987; Cancé et al., 1993), studies on the expression of lamins in heart tissue are sparse (Lehner et al., 1987; Röber et al., 1989; Lockard and Bloom, 1993). Lamin expression in human heart has as far as we know not been investigated. Here we report the changes in the expression of A- and B-type lamins in cardiomyocytes in biopsies from patients with chronic hibernating myocardium as compared to normal heart.

Materials and Methods

Patients

The human cardiac tissue material used in this study consisted of transmural biopsies obtained from 13 ischemic patients at the time of coronary artery bypass surgery. The biopsies used in the present study were obtained before any anastomoses were performed. All patients gave their informed consent. The study was approved by the local Ethical Committees for Research. The detailed individual patient characteristics are described previously (Maes et al., 1994). In short, 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 months after coronary bypass surgery. Eight patients had, as demonstrated by PET, a flow-metabolic mismatch pattern in their hypokinetic segments. From the other five patients, biopsies from normokinetic segments were used as controls.

Light microscopic evaluation

Of all patients a first biopsy was fixed for a minimum of 2 h in 3% glutaraldehyde, buffered to pH 7.4 with 90 mM KH₂PO₄, washed in the buffer supplemented with 220 mM sucrose and post-fixed for 1 h in 2% OsO₄, buffered with 50 mM veronal acetate, dehydrated in a graded series of ethanol and embedded in epoxy resin (Flameng et al., 1984). Epon sections (2 μm thick) were used for light microscopic morphometry of endo- and epicardial parts of the myocardium stained with either 0.1% toluidine blue or periodic acid-Schiff (PAS). This staining method permits independent visualization of the contractile elements and glycogen respectively and enables the unambiguous determination of the degree of myolysis. The effect of autolytic changes which could be responsible for degradation of glycogen, should be negligible since tissues were fixed immediately after the biopsy had been taken. In order to evaluate cellular changes in the different layers of the myocardium, at least 100 myocardial cells per segment were analysed. Only cells in which the nucleus was visible in the plane of observation were included in the analysis. Cells were planimetrically scored for the degree of myolysis (sarcomere loss). Since space, formerly occupied by sarcomeres, was mainly filled up by glycogen and mitochondria, quantitation of the changes was done on PAS stained sections. The percentage of glycogen content was planimetrically evaluated for each individual cell. If the sarcomere replacement accounted for more than 10%, cells were classified as affected.

The morphometric analysis of the amount of connective tissue in the myocardium was assessed using a grid with vertical and horizontal lines as previously described (Flameng et al., 1984). This method allows a quantitative analysis of the volumes of structures under investigation by counting the number of intersections overlying a certain structure. In this study, longitudinal sections at a...
magnification × 250 were evaluated. Blood vessels and perivascular interstitial cells were considered not to belong to the connective tissue.

Indirect immunofluorescence assays

A second biopsy of the hibernating myocardium was quickly frozen in isopentane pre-cooled with liquid nitrogen. Five-μm thick sections were air-dried, fixed in methanol (−20°C for 1 min), followed by acetone (4°C for three times 5 s), air dried again. 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 or goat-anti-mouse Ig-subclass specific antibody [Southern Biotechnology Associates (SBA) Inc., Birmingham, AL, USA], rabbit-anti-mouse antibody (DAKO A/S, Glostrup, Denmark) or goat-anti-rabbit antibody (SBA) 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 1:10000 with PBS. As a control, application of the first antibody was omitted.

For biotin-labeled lectin the same procedure was followed; in this case fluorescein isothiocyanate (FITC)-labeled avidin (Vector Laboratories, Burlingame, CA, USA) was used as conjugate.

All slides were examined with a Zeiss Axiohot microscope. Photographs of the immunofluorescence studies were taken with a Kodak Tri-X-pan film with a 400 ISO setting.

The percentage of A-type lamin positive cardiomyocyte nuclei were counted in sections stained with anti-A-type lamin antibodies and anti-desmin antibodies (to visualize the sarcomeres), according to the protocols described above.

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 Axiohot microscope (Carl Zeiss, Oberkochen, Germany).

The following monoclonal antibodies were used in this study: (1) LN43 directed against lamin B2 and not cross-reacting with lamin B1 (Bridger et al., 1993) which was a gift from Dr E. B. Lane (Dundee, UK). (2) 119D5F1 directed against lamin B1 and not cross reacting with lamin B2 (Machiels unpublished), kindly provided by Dr Y. Raymond (Montréal, Quebec, Canada). (3) 41CC4, directed against the rod domain of A type lamins A and C, kindly provided by Dr G. Warren (Heidelberg, Germany). 41CC4 is known to react more strongly with lamin C isoforms than with lamin A isoforms (Hozák et al., 1995; Machiels et al., 1995). (4) R27, directed against the rod domain of A-type lamins A and C (Zatloukal et al., 1992), kindly provided by Dr G. Krohne (Heidelberg, Germany). R27 is known to give a stronger reaction with lamin C isoforms than with lamin A isoforms (Hozák et al., 1995; Machiels et al., 1995). (5) 133A2, raised against the carboxyl terminus of 98 amino acids exclusively present in lamin A and kindly provided by Dr Y. Raymond. This antibody recognizes lamin A but not lamin C. Epitope mapping using several deletion mutants of lamin A showed that the amino acids 598-611 form the epitope by 133A2 (Hozák et al., 1995).

In addition, rabbit polyclonal antibody against vimentin, pVim (Ramaekers et al., 1983) and biotin-labeled lectin from Psophocarpus tetragonolous (Sigma Chemicals, St Louis, MO, USA), which binds specifically to endothelial cells of the human myocardium (Laitinen et al., 1990), were used.

Results

Structural changes in chronic hibernating myocardium

The histological and ultrastructural changes in chronic hibernating myocardium have been described in detail previously (Borgers et al., 1993; Vanoverschelde et al., 1993; Maes et al., 1994).
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Figure 1  Electron microscopy of nuclei of hibernating cardiomyocytes. (a) Detail of a hibernating cell with severe  
myolysis. The nucleus is surrounded by glycogen (g) and small mitochondria (m). The nuclear heterochromatin is  
uniformly dispersed over the nucleoplasm (× 11 500). (b) Detail of another hibernating cardiomyocyte showing patchy  
distribution of heterochromatin (arrows) (× 11 500).

Briefly, these cardiomyocytes were characterized by depletion of sarcomeres and accumulation of  
glycogen. The degree of myolysis varied amongst the patients: the replacement of contractile material  
by glycogen was limited to the vicinity of the nucleus in many cells, but in others glycogen comprised the bulk of cytoplasm, leaving only a few or no sarcomeres at the cell periphery. Cells were  
considered affected when more than 10% of the cell volume consisted of glycogen. The average percentage of affected cells was 23 ± 10 for the group of eight patients examined in the present  
study, with a PET mismatch pattern. A very characteristic alteration during chronic hibernation  
concerned shape changes of the nuclei. These were often enlarged and showed a uniform dispersion  
of their heterochromatin material throughout the nucleoplasm [Fig. 1(a)], thereby resembling nuclei of cardiomyocytes during late developmental stages. However, a number of nuclei retained an apparently  
normal distribution pattern of heterochromatin in otherwise severely affected cells [Fig. 1(b)]. Since  
the data on glycogen and contractile elements have been obtained at the light microscopic level and  
those of the shape of the nuclei at the electron microscopic level these results have to be interpreted  
with caution and only indirect correlations can be suggested.

Lamin expression in human myocardium

An overview of the reactivity patterns of normokinetic and hibernating heart segments with  
different lamin monoclonal antibodies is given in Table 1 and depicted in Figures 2-4.

B-type lamin expression in normokinetic and hibernating heart sections

The normokinetic and hibernating heart sections showed identical staining patterns for B-type lamins. Lamin B2 staining was present in all nuclei throughout the sections [Fig. 2(a) and (b)]. Identical  
staining intensities were seen in the nuclei of the different myocardial cell types. However, the other  
lamin B subtype, i.e. lamin B1 was absent from virtually all cell types present in the heart. Cardiomyocytes were never observed positive for lamin
Table 1  Summary of immunohistochemical results for the lamin-subtypes in normokinetic, chronic hibernating and embryonic muscle cells

| Lamin subtype | Antibody | Normokinetic | Chronic hibernating | Embryonic *
|---------------|----------|--------------|---------------------|-------------
| A             | 133A2    | +            | +/- or −−           | −           |
| A/C           | 41CC4 and R27 | +            | +/- or −−           | −           |
| B1            | 119D5-F1 | −            | −                   | −           |
| B2            | LN43     | +            | +                   | +           |

* literature data (refs Lehner et al., 1987; Röber et al., 1989; Lourim and Lin, 1989), concerning results from other species, i.e. chicken, mice and Xenopus
+ positive; +/- partly or weakly positive; − negative.

Figure 2  Immunofluorescence micrographs of lamin B subtypes of different sections of human heart. Nuclear lamina were shown to lamin B2 positive (a) and nuclei were stained with DAPI (b) (× 570). (c) Nuclear lamina showed no staining with lamin B1 (c), nuclei are visible by DAPI staining (d) (× 500). Arrows indicate autofluorescence of lipofuscin.

B1 [Fig. 2(c) and (d)] nor were chronic hibernating segments that displayed myolysis. The few lamin B1-positive cells were identified as mesenchymal cells on basis of their positive staining for vimentin. Double labeling studies with lectin and vimentin showed that some but not all of the endothelial and fibroblastic cells were positive for lamin B1.

A-type lamin expression in normokinetic heart sections

All cardiomyocytes in normokinetic heart sections showed A-type lamin expression, with only the nuclear periphery stained with all three A-type monoclonal antibodies. Vascular smooth muscle cells [Fig. 3(a) and (b)] and interstitial cells were
also observed to be positive for A-type lamins, although the reactivity was variable and occasionally absent. No differences were observed in staining intensity with the different monoclonal antibodies against A-type lamins.

A-type lamin expression in hibernating heart sections

Nuclei of cardiomyocytes of chronic hibernating heart segments showed a decrease in staining intensity with the monoclonal antibodies against the different lamin A-subtypes. A considerable number of cardiomyocyte nuclei were found to be negative for A-type lamins [Fig. 3(c) and (d)]. In average about 80% of nuclei in myolytic (hibernating) cardiomyocytes lack A-type lamin staining. With double labeling studies subtle differences in the staining intensity were sometimes seen with the different monoclonal antibodies against A-type lamins. The monoclonal antibody 133A2 (specific for lamin A) showed a more positive staining than R27 and 41CC4 (recognizing both lamins A and C) in nuclei of chronic hibernating cardiomyocytes. The DAPI staining which was routinely included in the analyses did not show signs characteristic for apoptosis.

Internuclear distribution of lamins

In order to investigate the organization of A-type lamins in nuclei of chronic hibernating cardiomyocytes immunofluorescently stained sections were analysed by confocal laser scanning microscopy. Although, most myolytic cardiomyocytes were negative for A-type lamins (80%) [Fig. 4(a)], some displayed these lamins (20%). In several of these cells A-type lamin staining was seen as patches over the whole nucleoplasm instead of being concentrated at the border of the nucleus [Fig. 4(b)]. In contrast, normal cardiomyocytes displayed A-type and B-type lamin staining at the border of the nuclei [Fig. 4(c)].

Figure 3 Immunofluorescence micrographs of A-type lamin expression in heart muscle. (a,b) Staining of a bloodvessel with A-type lamins (41CC4) (a) and DAPI nuclear staining (b) (440). (c,d) Staining of A-type lamins (41CC4) (c) and DAPI nuclear staining (d) in a section from chronic hibernating myocardium. Nuclei form hibernating cardiomyocytes were shown to be negative for A-type lamins. Dot-like staining is autofluorescence of lipofuscin ( × 500).
Figure 4  Confocal laser scanning microscopy of nuclei seen in chronic hibernating segments. Double labeling studies of A-type lamins (green) with lamin B2 (red). (a) Absence of A-type lamins staining, while the contour of the nucleus is visible with lamin B2 staining, as seen in approximately 80% of the cardiomyocytes with myolysis (× 1850). (b) Patchy A-type lamin staining throughout the nucleoplasm of a nucleus with an irregular contour from a myolytic cardiomyocyte. This pattern is seen in a minor fraction of the myolytic cardiomyocytes (× 1850). (c) A-type lamin and lamin B2 staining at the border of a nucleus from a normal cardiomyocyte. Large red dots which are visible in the perinuclear area represent lipofuscin autofluorescence (× 2056).

Discussion

Cardiomyocytes from chronic hibernating myocardium display typical subcellular alterations such as loss of myofibrils, accumulation of glycogen, fragmentation of sarcoplasmic reticulum, the presence of strands of rough endoplasmic reticulum, loss of T-tubular structures and shape changes in their mitochondria (Borgers et al., 1993). Some of these changes are cellular features of dedifferentiation. In addition, the expression pattern of contractile and cytoskeletal proteins such as titin, cardiotin and α-smooth muscle actin resembles that of embryonic cardiomyocytes (Ausma et al., 1995a,b). Nuclei of chronically hibernating cardiomyocytes are often enlarged and many of them demonstrate a uniform dispersion of their heterochromatin in a way similar to that observed during embryonic development. Nuclear A-type lamins are known to be expressed in a differentiation-related fashion and contribute to nuclear integrity and chromatin organization. A-type lamin expression is absent in undifferentiated cells or cells at early stages of differentiation (Stick and Hansen, 1985; Stewart and Burke, 1987; Lehner et al., 1987; Paulin-Levassere et al., 1988; Röber et al., 1989; Guilly et al., 1990). In the present study, we therefore investigated the expression patterns of different nuclear lamin subtypes in connection with the hypothesis that chronic hibernating myocardium exhibits dedifferentiation characteristics.

The expression of A-type lamins in normal and chronic hibernating myocardium

Our results obtained with monoclonal antibodies directed against A-type lamins indicate the loss of lamins A and C in 80% of the cardiomyocytes displaying myolysis. Since we have used A-type lamin antibodies recognizing different epitopes on both molecules, it can be excluded that these findings result from epitope masking or partial breakdown of the lamins. Our results thus indicate that the expression of both A-type lamins is absent as a result of their hibernating state, providing additional proof for their dedifferentiated phenotype. In particular the fact that lamin B2 remains present in cells that lack A-type lamins supports the idea that the myolytic cells are not subjected to degeneration. In such processes both A- and B-type lamins disappear simultaneously (Oberhammer et al., 1993; Tinnemans et al., 1995).

We realize that caution must be taken when correlating lamin expression in cardiomyocytes during chronic hibernation in man with that during cardiac differentiation, since expression of different lamin subtypes during development of human cardiac muscle has not yet been documented. A-type lamin expression did not occur during myofibrillogenesis in chicken and mouse before the cells have committed to the myogenic differentiation pathway, as indicated by the expression of several specific muscle cell markers. A-type lamin expression preceded, however the induction of late...
nuclear lamina may be important in establishing lar heterochromatin in hibernating car-
the nuclear localization signal. Transfections with
terogenic cells with lamin A, or lamin A lacking
established by transfection of chick embryonic my-
chromatin topology. The role of lamin A was es-
bryonic phenotype.

A-type lamins are assumed to exert lamins. These phenomena may exert an effect on
differentiation pathways. Apparently, the presence of
other nuclei, a more patchy heterochromatin dis-
ferentiation gene expression in these cells resulting in an em-
byronic phenotype.

The absence A-type lamin staining observed in a plication of the absence of lamin B1 remains to be
result of chronic hibernation.

However, our preliminary studies on the
ubiquitous expression of lamin B1 and lamin B2 is
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self study shows that all cardiac muscle cells,
including the hibernating ones, were lamin type
B1 negative. Although all nuclei of cardiac cell
types were shown to be positive for lamin B2,
only a small percentage of the interstitial cells
(fibroblasts, pericytes and endothelial cells) were
shown to be lamin B1 positive.

So far, the expression of B-type lamins has been
thought not to be developmentally regulated or to
be associated with the state of differentiation (Stick
and Hansen, 1985; Paulin-Levasseur et al., 1988; Röber et al., 1989; Guilly et al., 1990). The only
report showing a differential expression of both B-
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1992). However, our preliminary studies on the
presence of lamins B1 and B2 in several human
tissues indicate that a differential expression of these
two B-type lamins, especially the absence of lamin
B1, occurs more often than previously assumed
(Broe et al., unpublished). The biological im-

References

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Tropomyosin (Lourim
etc., 1989; Guilly
et al., 1989; Lockard and Bloom,
1990). The only Internal lamin substructure within G1 nuclei of human
changes of titin in left ventricular dysfunction as a result of chronic hibernation. J Mol Cell Cardiol 27:1203-1212.
myocardial dysfunction in patients with critically cor-
BURKE B, GERACE L, 1986. A Cell free system to study reassembly of the nuclear envelope at the end of
BRIDGER JM, KELL J, O’FARRELL M, HUTCHISON CJ, 1993. Internal lamin substructure within G1 nuclei of human
angiographic histologic and electrocardiographic data

The expression of B-type lamins in normal and chronic
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