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Adult rabbit cardiomyocytes undergo hibernation-like dedifferentiation when co-cultured with cardiac fibroblasts

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Abstract

Objectives: Little is known about the causal factors which induce the typical structural changes accompanying cardiomyocyte dedifferentiation in vivo such as in chronic hibernating myocardium. For identifying important factors involved in cardiomyocyte dedifferentiation, as seen in chronic hibernation, an in vitro model mimicking those morphological changes, would be extremely helpful. **Methods:** Adult rabbit cardiomyocytes were co-cultured with cardiac fibroblasts. The typical changes induced by this culturing paradigm were investigated using morphometry, electron microscopy and immunocytochemical analysis of several structural proteins, which were used as dedifferentiation markers, i.e., titin, desmin, cardiotin and α -smooth muscle actin. **Results:** Close apposition of fibroblasts with adult rabbit cardiomyocytes induced hibernation-like dedifferentiation, similar to the typical changes seen in chronic hibernation in vivo. Both changes in ultrastructure and in the protein expression pattern of dedifferentiation markers as seen in chronic hibernating myocardium were seen in the co-cultured cardiomyocytes. **Conclusion:** Hibernation-like changes can be induced by co-culturing adult rabbit cardiomyocytes with fibroblasts. This cellular model can be a valuable tool in identifying and characterizing the pathways involved in the dedifferentiation phenotype in vivo, and already suggests that many of the structural changes accompanying dedifferentiation are not per se dependent on a decreased oxygen availability. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Cell culture/isolation; Hibernation; Histo(patho)logy; Electron microscopy; Remodeling

This article is referred to in the Editorial by G.C. Hughes (pages 191–193) in this issue.

1. Introduction

Patients with viable hypocontractile myocardium and delayed functional recovery after reperfusion (chronic hibernating myocardium) show typical cellular and extracellular structural abnormalities. The most important cellular alterations are a redistribution of nuclear heterochromatin, depletion of sarcomeres, aberrantly shaped but healthy mitochondria and a degradation of structured sarcoplasmic reticulum in the cardiomyocytes [1–3]. These changes give rise to cardiomyocytes with the structural

hallmarks of fetal heart cells (dedifferentiated state) and are believed to be at least partially involved in delayed functional recovery after reperfusion [4,5]. The expression pattern of several structural proteins resembles that of embryonic/fetal cardiomyocytes. The highly organized patterns of cardiotin, titin and desmin disappear, while there is a re-expression of α -smooth muscle actin. Recently, however, it became clear that these changes are not an exclusive feature of chronic hibernating myocardium, but can also occur in other pathological situations as in chronic fibrillating atria [6], infarction border zones [7], and pressure-overloaded myocardium [8]. The mechanisms underlying the phenotypic changes are unknown, but recent findings suggest that in the setting of hibernation, the oxygen shortage due to chronic or repetitive underperfusion accompanied by limited flow reserve, may be the

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initiating but not the intrinsic causal factor. Indeed, in animal models of both acute [9] and chronic [10] hibernation, structural changes were not only limited to the underperfused areas, but were also observed in remote, normally perfused regions. However, unraveling a 'cause-effect' relationship *in vivo* is often complicated. Therefore, cultured cardiac myocytes can be extremely helpful in efforts to directly identify and characterize putative pathways leading to hibernation-like dedifferentiation. Previous investigations have indicated that the choice of an appropriate cellular model (neonatal/adult, species, culture conditions) is of utmost importance when pathways leading to altered myocyte growth are to be identified. For example, adult rat cardiomyocytes in culture display spontaneous dedifferentiation and partial redifferentiation very quickly after acquiring spontaneous contractile activity [11–13]. However, these structural changes are only partially comparable to the morphological changes seen in dedifferentiation *in vivo* [14]. Moreover, in order to get more insight in putative triggers, it would be of interest to find a culture system in which dedifferentiation could be induced, instead of occurring spontaneously. It was the aim of this study to develop a cellular model of isolated cardiac myocytes in which the morphological changes of dedifferentiation *in vivo* could be mimicked. It was previously reported that the growth characteristics of cultured cardiomyocytes could be influenced by non-myocytes and by growth factors [14,15]. Therefore we investigated the influence of co-culturing adult rabbit cardiomyocytes with mesenchymal cells (mainly fibroblasts). Since morphological changes are the best characterized pathophysiological features of chronic hibernating myocardium, we focused on comparing the morphological characteristics of the myocytes in the culture system with those of hibernating myocytes in patients by using immunocytochemistry and electron microscopy. We found that by co-culturing adult rabbit cardiomyocytes and cardiac mesenchymal cells (mainly fibroblasts), the typical ultrastructural changes and changes in protein expression patterns of dedifferentiation *in vivo*, could be induced in the cultured myocytes. Therefore, this cellular model can be a valuable tool in identifying and characterizing pathways that control the typical morphological changes of the 'hibernating' phenotype of cardiomyocytes in different cardiac pathologies.

2. Methods

2.1. Cell isolation and culture conditions

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Cardiomyocytes and cardiac fibroblasts were isolated from adult NZ white rabbits (ca. 2 kg) by retrograde collagenase perfusion. Rabbits were anesthe-

tized by intra-arterial injection of pentobarbital (Sanofi, Brussels, Belgium; 70 mg/kg), the heart was rapidly excised and perfused in a retrograde Langendorff mode (5 min — 35 ml/min) with a modified calcium-free Krebs–Henseleit buffer (37°C) containing (in mM): NaCl 125, KCl 2.5, KH₂PO₄ 1.5, MgSO₄ 1.2, Hepes 10, glucose 9.5, pyruvate 5, gassed with 100% O₂. Then, fresh buffer, supplemented with 0.6 mg/ml collagenase (Wako, Osaka, Japan), 1 mg/ml BSA and 2.5 μM Ca²⁺, was recirculated for 40 min. The Ca²⁺ concentration was increased to 75 and 175 μM after 13 and 18 min, respectively. The ventricles were gently dissociated in the same medium without collagenase and the cells were filtered through a 200-μm² mesh. In three subsequent rinsing steps, with the buffer supplemented with 10 mg/ml BSA, the Ca²⁺ concentration was elevated to 1.4 mM. The cells were suspended in Medium 199 (Life Technologies, Paisley, Scotland) supplemented with antibiotics (penicillin–streptomycin 0.1 mg/ml; gentamycin 2 μg/ml) and seeded at a low density to prevent cell–cell contact (10³ cells/cm²) on laminin-coated (10 μg/ml; Life Technologies) cover-glasses in petri dishes, and allowed to attach for 2 h. Thereafter, fresh medium supplemented with 20% fetal bovine serum (FBS, Hyclone, UT, USA) was applied to the cells. Cells were kept in a humidified CO₂ incubator (5% CO₂, 37°C) for up to 21 days, and medium was replaced twice a week. In parallel experiments, 10 μM cytosine-1-β-D-arabinofuranoside (AraC, Sigma) was added to the culture medium to inhibit proliferation of non-myocytes. Fibroblast-conditioned medium (FCM) was collected by growing cardiac fibroblasts to a sub-confluent state. Fresh medium as described above was applied to these cultures and the cells were again collected after 24 h. To investigate the influence of secreted factors, FCM was applied to fresh cardiomyocyte cultures and replaced daily. Alternatively, cardiac fibroblasts were grown on cell inserts (0.02 μm Anapore, Nunc, Life Technologies) which were applied to petri dishes seeded with freshly isolated cardiomyocytes.

2.2. Morphometric analysis

Individual cardiomyocytes were followed at regular time intervals by use of an Axiovert microscope in phase contrast illumination mode (Carl Zeiss, Oberkochen, Germany), and equipped with a CCD camera. Images were captured and used for morphometric analysis. Cardiomyocytes were outlined and the cell surface area was measured with the software package SCIL-Image version 1.4 (TNO-TPD, Delft, The Netherlands) on an O2 workstation (SGI, Mountain View, CA).

2.3. Immunocytochemistry

Cells were fixed immediately after isolation (in suspension), a few hours after seeding (day 0, d0), and then every

other day starting at day 1 (d1) and ending at d21. The medium was discarded and the cells were rinsed with phosphate-buffered saline (PBS). Three fixation protocols were used. For the analysis of all structural proteins except cardiotin and α -smooth muscle actin, the cells were fixed by cold (-20°C) methanol (3×1 s)–acetone (1×1 s) treatment and air dried. These cells were kept at -20°C until processed further. For determination of cardiotin, the cells were briefly fixed (10 min) in 4% paraformaldehyde at room temperature. The cover-glasses were then rinsed in PBS and AD, air dried, and frozen to -20°C until further use. For the analysis of α -smooth muscle actin expression, the cells were fixed in cold (4°C) 3% glutaraldehyde in 0.09 M KH_2PO_4 + 1.4% sucrose (pH 7.4) for 20 min. The cover-glasses were rinsed with the buffer and AD, air dried, and then frozen to -20°C .

The following primary antibodies, which have been described previously [6], were used: a mouse monoclonal antibody 9D10 to titin (diluted 1:10), a mouse monoclonal antibody RD301 to desmin (undiluted), a mouse monoclonal antibody R2G to cardiotin (undiluted), a mouse monoclonal antibody EA-53 to α -actinin (Sigma) and a mouse monoclonal antibody SM-1 (1:100) to α -smooth muscle actin (Dako, Glostrup, Denmark).

The following protocol was used for immunofluorescent labeling: the cells were thawed, rinsed with PBS and then incubated with 0.1% Triton X-100 (Sigma) in PBS. After being rinsed in PBS+0.5% bovine serum albumin (BSA, Sigma) ($3\times$), the cells were incubated with the primary antibody for 1 h at room temperature. The secondary Cy³-labeled goat anti-mouse antibody (Jackson Immuno-research, West Grove, USA) was applied for 45 min after three rinsing steps in PBS+0.5% BSA. The cover-glasses were rinsed in PBS and f-actin was stained with phal-

loidin-FITC (Sigma). The glasses went through a final rinsing step in PBS and AD and then mounted with Prolong[™] antifade reagent (Molecular Probes, Leiden, The Netherlands). The cells were visualized by use of a confocal laser scanning microscope (LSM 510, Carl Zeiss). For α -smooth muscle actin staining, the cells were pre-treated with 50 mM NH_4Cl for 10 min and with 0.2% Triton X-100 (Sigma) for 5 min. The cover-glasses were incubated overnight at room temperature with the primary antibody diluted in PBS with 0.05% Tween 20 (Sigma), and then rinsed (3×5 min with PBS) and incubated for a further 45 min with the secondary antibody (biotinylated anti-mouse, Vector Laboratories, Burlingame, USA) diluted in PBS with 0.05% Tween 20. The Vectastain ABC-kit (alkaline phosphatase, Vector) together with Vector Blue chromogen (Vector) were used according to the manufacturers instructions for visualizing the immunogen, before the cover-glasses were mounted with Kaiser's glycerol gelatin (Merck, Darmstadt, Germany).

2.4. Electron microscopy

For electron microscopic evaluation of the phenotypical changes, cells were fixed in 3% glutaraldehyde buffered to pH 7.4 with 90 mM KH_2PO_4 and KOH, at regular intervals between d0 and d21. After post-fixation with 2% OsO_4 in 50 mM veronal acetate (1 h), the cells were dehydrated in graded series of ethanol, and embedded in Epon epoxy resin according to standard procedures. Ultrathin sections were counterstained with uranium acetate and lead citrate, prior to examination in a Philips CM100 electron microscope.

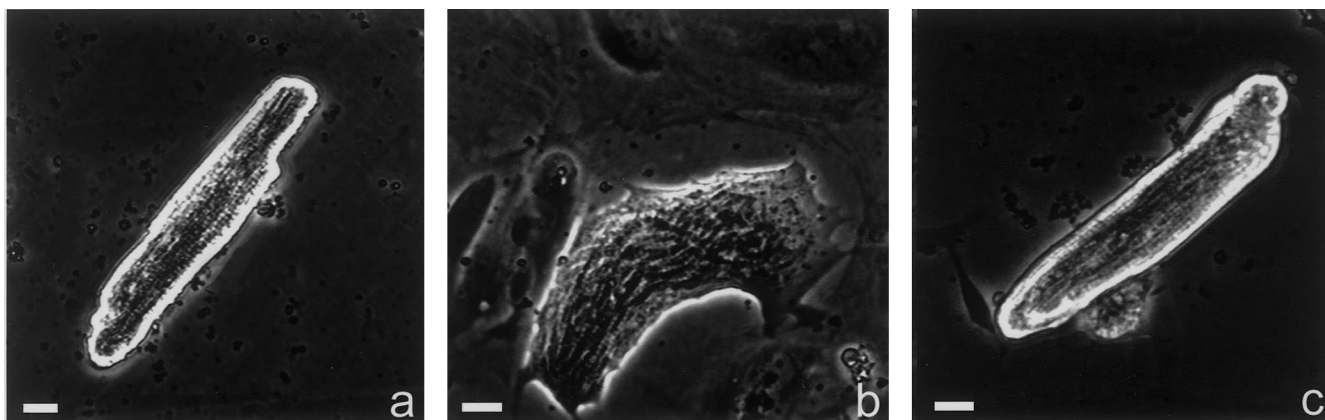
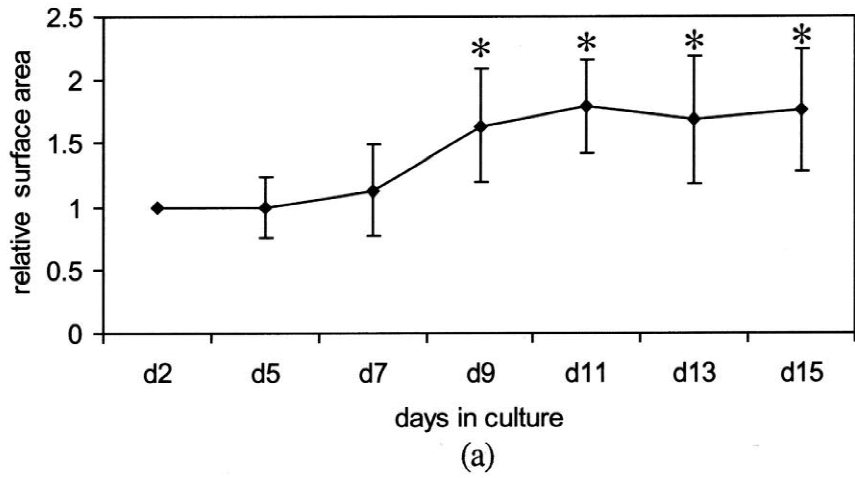


Fig. 1. Phase contrast images of adult rabbit cardiomyocytes in co-culture (bars represent 10 μm). (a) A cardiomyocyte 2 days after isolation and before close apposition with fibroblasts. The cell is rod-shaped and displays obvious cross-striations. The only obvious change compared to freshly isolated cardiomyocytes is the rounding of previously staircase-like cell ends. (b) A cardiomyocyte, 7 days in culture, spread after close apposition with fibroblasts. Cell surface area increased, but the basic elongated shape of the cell is not lost; however, the cross-striated pattern is somewhat disaligned. (c) After 7 days in culture, an adult rabbit cardiomyocyte in araC-supplemented cultures is still rod shaped and indistinguishable from cardiomyocytes in day 2 cultures without araC.



Cardiomyocyte surface area

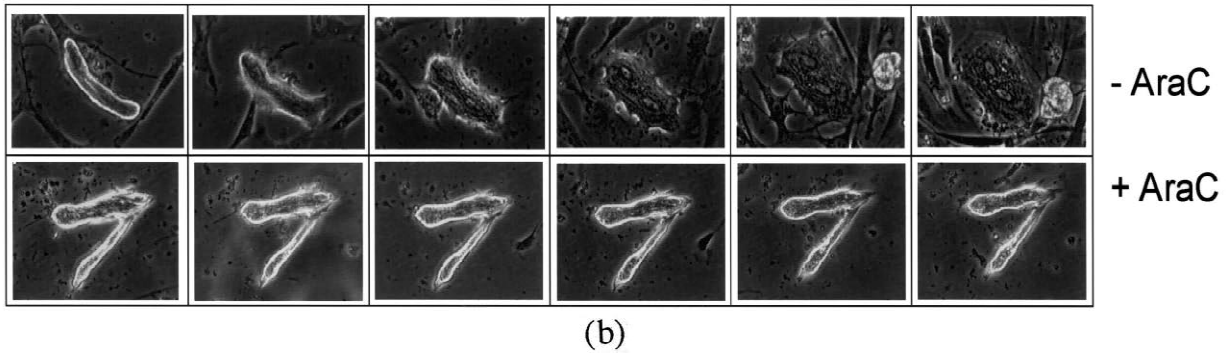
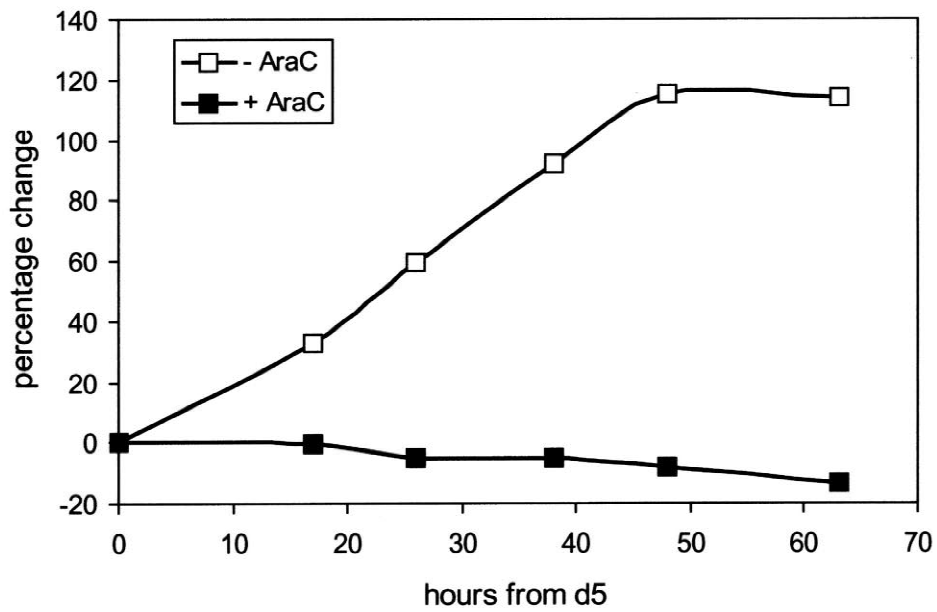
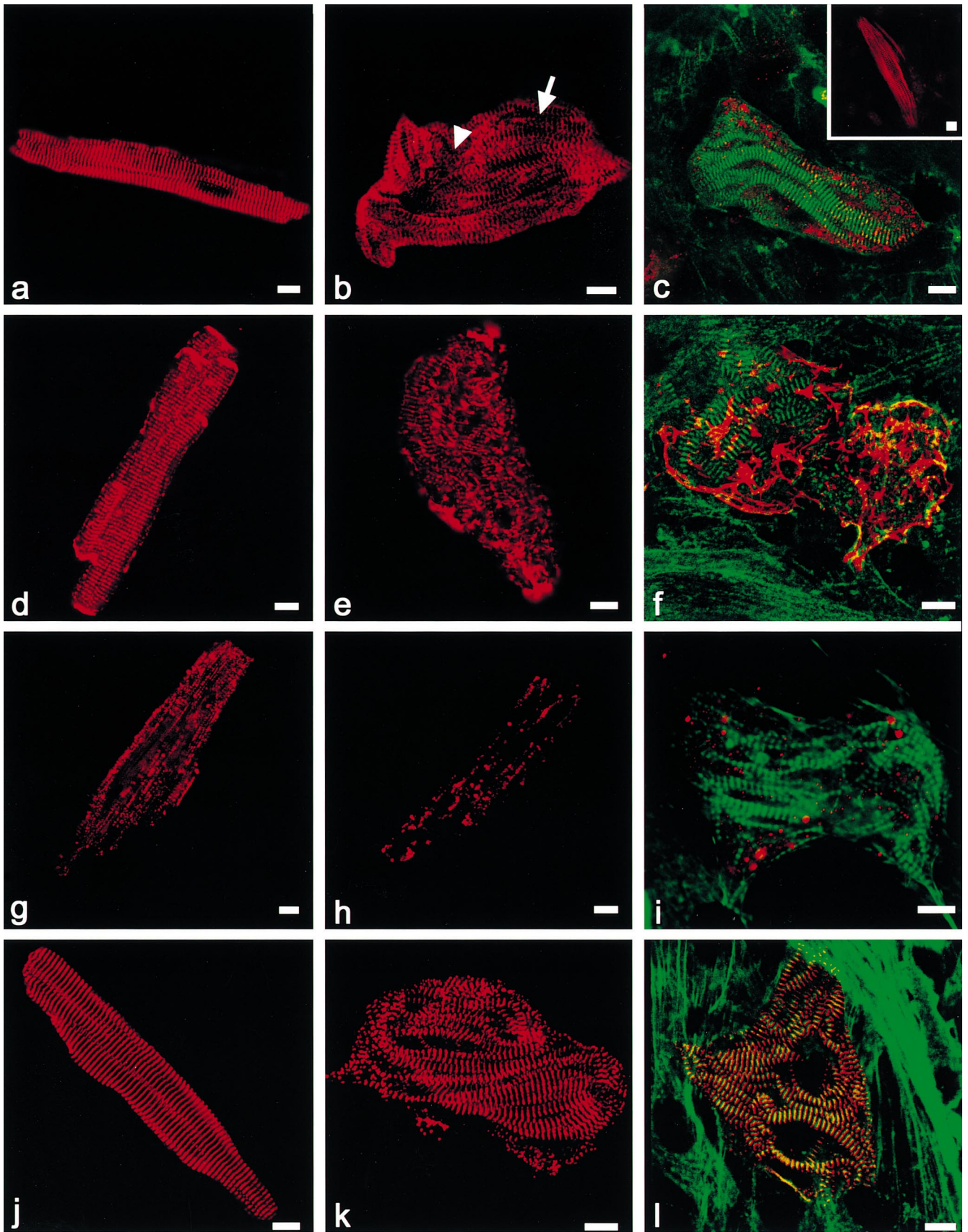


Fig. 2. (a) Relative increase in cell surface area of co-cultured adult rabbit cardiomyocytes. (* $P < 0.05$ versus d2). (b) Relative increase in cell surface area between d5 and d7 of cardiomyocytes in fibroblast-rich (-AraC) and fibroblast-poor (+AraC) conditions.



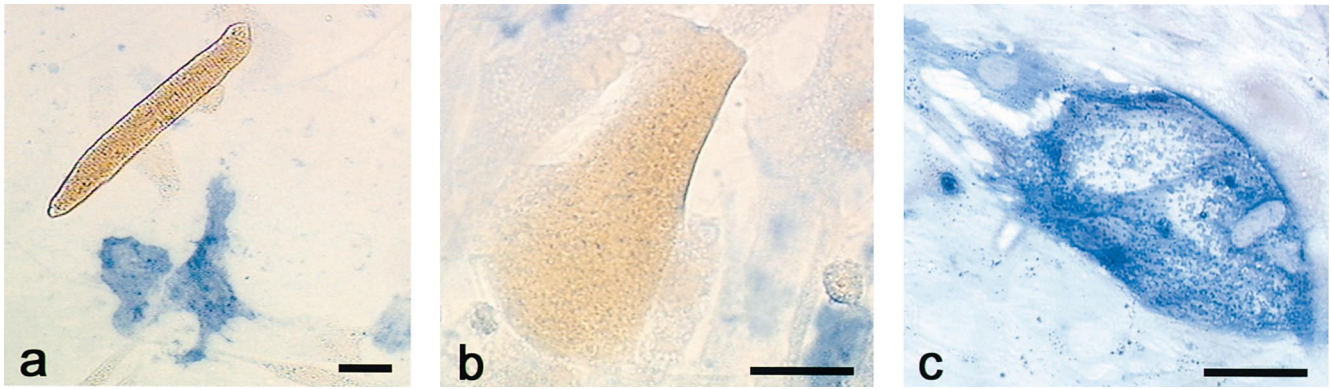


Fig. 4. Immunocytochemical detection of α -smooth muscle actin (bars represent 50 μm). (a) Cardiomyocytes 4 days in co-culture do not express α -smooth muscle actin, unlike some of mesenchymal cells, possibly endothelial cells. (b) After 10 days in co-culture, cardiomyocytes are still negative for α -smooth muscle actin. (c) Some of the more dedifferentiated cardiomyocytes (less detectable cross-striations) after 15 days in co-culture re-express α -smooth muscle actin. Only a diffuse staining could be observed, and no organization into filaments is seen.

3. Results

3.1. Morphological changes

After attachment to the laminin-coated surface, the adult rabbit cardiomyocytes remained rod-shaped during the early days of culture (Fig. 1a). The only obvious change that could be noted with the light microscope was a rounding of the previously staircase-like distal ends of the cell. In contrast to the myocytes, the interstitial cells (mainly fibroblasts) proliferated. Because of this proliferation, close appositions between myocytes and fibroblasts became evident from day 3 onwards and increased over time. Rapid morphological changes of the myocytes occurred soon after cell–cell apposition between fibroblasts and cardiomyocytes was established. The first obvious change was a flattening of the myocyte (Fig. 1b), with an increase in cell surface area, but without the loss of its elongated shape, i.e., no rounding was observed and no pseudopodia-like structures became evident. The mean increase in cell surface area, when cells of d10 were compared with cardiomyocytes of d2 was $39.6 \pm 1.7\%$ (mean \pm S.E.M.). In contrast to myocytes in close apposi-

tion with fibroblasts, cardiomyocytes that were not in close apposition with the fibroblasts did not display this flattening, and retained their normal rod-shaped appearance until contact became evident. Myocyte spreading was maximal between d7 and d9, when myocyte cell surface area reached a plateau (Fig. 2a). In contrast, d7 myocytes in araC-supplemented cultures were still rod-shaped as fresh isolated cells (Figs. 1c, and 2b). In d7 cultures, virtually all myocytes were in close apposition with fibroblasts, and thus all displayed morphological changes, meaning that it took approximately 4 days between the first changes occurring and the moment when almost all myocytes had spread. Within the same time frame, such typical flattening of the cells could not be induced either by application of FCM on freshly isolated cardiomyocytes, or by use of fibroblast-grown cell inserts in petri dishes with freshly isolated myocytes.

3.2. Distribution pattern of structural proteins

3.2.1. Titin

In freshly isolated cardiomyocytes, the 9D10 antibody showed a typical cross-striated pattern (Fig. 3a). This

Fig. 3. Immunocytochemical detection of dedifferentiation markers (bars represent 10 μm). (a) Normal cross-striated titin distribution in freshly isolated cardiomyocytes. (b) Co-cultured cardiomyocyte after 7 days. The first obvious changes in the titin pattern of the cardiomyocytes are, apart from the loss of alignment of the sarcomeres, a decreased staining intensity in some areas (arrow) and a loss of the normal cross-striated pattern (arrowhead). (c) After almost 3 weeks in co-culture, the 9D10 epitope of titin is almost completely lost. Only some unorganised dotlike structures can be detected (in red); however, some sarcomere organisation can still be seen with phalloidin-FITC (in green). Nevertheless, in fibroblast poor conditions (araC-treated cultures) a virtually normal titin pattern remains present in cardiomyocytes (insert). (d) In freshly isolated cardiomyocytes desmin shows a normal cross-striated pattern next to a localisation at the distal cell ends of the former intercalated disks. (e) After 7 days in co-culture, the cross-striated desmin pattern is lost and spread out over the cytoplasm so is its localization at the distal cell ends. (f) The normal desmin pattern is redistributed in cardiomyocytes co-cultured for 10 days. Desmin is now present in a filamentous pattern throughout the cell (in red), which will gradually disappear in the cell center, leaving only a network at the cell periphery. Remaining sarcomeres can be observed with phalloidin-FITC (in green). (g) The R2G antibody against cardiotin recognizes longitudinally oriented punctate arrays in freshly isolated cardiomyocytes. (h) Already after 3 days the longitudinal arrays of cardiotin have shortened, leaving only dotlike structures. (i) Small dots remain detectable with the R2G antibody in a co-cultured cardiomyocyte after 15 days of co-culturing. (j) A cardiomyocyte 1 day in culture displays a normal cross-striated α -actinin pattern. (k) Although sarcomeres become disarranged after 7 days in co-culture, the α -actinin pattern remain normally cross-striated. (l) In remaining sarcomeres, α -actinin remains present in a normal cross-striated fashion, after 12 days in co-culture.

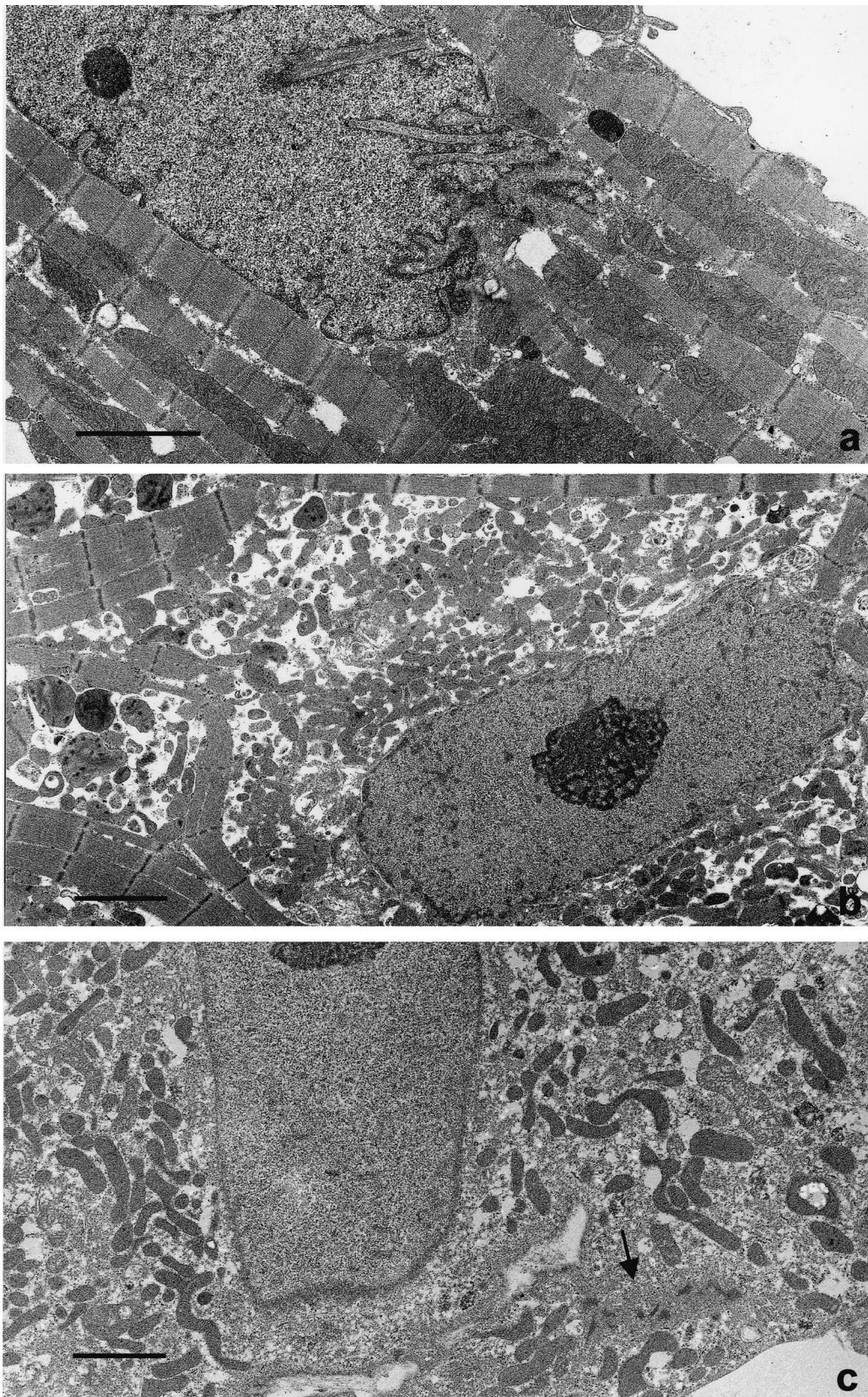


Fig. 5. Electron microscopy of co-cultured adult rabbit cardiomyocytes (bars represent 1 μm). (a) Ultrastructural appearance of an adult rabbit cardiomyocyte during the first day of culture. Aligned intact sarcomeres are present throughout the cytosol. Normal mitochondria are aligned between the sarcomeres. The heterochromatin in the nucleus is clustered near the nuclear membrane. (b) Adult rabbit cardiomyocyte after 8 days in co-culture. Severe sarcomere depletion and disalignment is obvious, and so is the appearance of abnormally shaped (small and elongated) mitochondria. Nuclear heterochromatin shows a patchy distribution. (c) After 13 days in co-culture, cardiomyocytes can only be identified by the presence of small sarcomere remnants (arrow). Numerous small and elongated mitochondria can be noticed. The nucleus displays a homogeneous dispersion of heterochromatin.

normal pattern remained present in the following days, as long as the myocytes were not interacting with fibroblasts.

The first change that became obvious in the titin pattern paralleled the final stage of flattening of the myocyte after interaction. During the flattening, not only did the sarcomeres lose their regular alignment, but there were areas in which the 9D10 epitope (A–I junctional part of titin) showed a decreased intensity (Fig. 3b). These changes were obvious in most myocytes after d7 in co-culture. In the following days the decrease became more pronounced, but seemed to parallel a decrease in sarcomere density. However, after about 3 weeks of co-culture it was obvious that the 9D10 staining was reduced to a punctate pattern and there were only a few small regions with a recognizable cross-striation, while sarcomeres could be observed with phalloidin-FITC (Fig. 3c). That the changes were triggered by fibroblast involvement was confirmed by the finding of a virtually normal titin pattern in d21 cardiomyocytes in araC supplemented cultures (Fig. 3c, insert).

3.2.2. Desmin

The intermediate filament-type protein desmin could be observed in a normal cross-striated distribution pattern in freshly isolated cardiomyocytes. Heavy staining was noted at the distal ends of the cell, at the site of the former intercalated disk (Fig. 3d). This pattern stayed stable until myocyte–fibroblast interaction was obvious. The first changes in the RD301 staining pattern were seen to coincide with the start of the spreading of the myocytes: a decreased staining intensity at the distal cell endings, and a kind of smearing out of the normal cross-striated pattern (Fig. 3e). Between d7 and d10, the desmin cross-striation was almost completely lost, and transforming into a filamentous pattern throughout the cell. These changes preceded a gradual decrease in the density of myofibrils. The filamentous desmin pattern gradually disappeared in the cell centre, leaving only an irregular desmin network at the cell periphery after 3 weeks of co-culture (Fig. 3f).

3.2.3. Cardiotin

The R2G antibody against this protein recognized longitudinally oriented structures (Fig. 3g) perpendicular to the cross-striations in freshly isolated cardiomyocytes.

However, this pattern rapidly disappeared soon after the myocytes had attached. These arrays had already become shorter by d1 in culture. On d3, the normal cardiotin pattern had completely changed to a punctate pattern, with no more than some short arrays remaining in some cells (Fig. 3h). Interaction with fibroblasts seemed not to be a prerequisite for this change in R2G epitopes. Only small dots remained randomly distributed in the cells, visible throughout the rest of the culturing period (Fig. 3i).

3.2.4. α -Actinin

The normal cross-striated pattern, as can be seen in freshly isolated cardiomyocytes (Fig. 3j) was detectable in remaining sarcomeres for the whole culturing period, and therefore reflected the changes in sarcomere alignment and sarcomere density. From the moment myocytes began to flatten, sarcomere alignment became disrupted (Fig. 3k). This was followed by a gradual decrease in sarcomere density and length, and after 3 weeks of co-culture, the myocytes showed only some remnants of sarcomeres displaying a normal cross-striated α -actinin pattern (Fig. 3l).

3.2.5. α -Smooth muscle actin

Within the first 2 weeks of co-culture, only some interstitial cells, presumably endothelial cells and smooth muscle cells, stained positive with the antibody to α -smooth muscle actin. The cardiomyocytes were negative without exception (Fig. 4a). However, some myocytes displayed diffuse staining after this period (Fig. 4b). These positive cells had only few sarcomeres left, whereas other myocytes with more sarcomeres — most probably in an earlier stage of dedifferentiation — were negative. No structural organization of α -smooth muscle actin into filaments was seen.

3.3. Electron microscopy

At the ultrastructural level, prior to fibroblast contact, isolated myocytes showed a typical highly organized sarcomere structure, with uniformly sized mitochondria in between these sarcomeres. The cardiomyocyte nuclei contained a typical heterochromatin distribution in small clusters at the nuclear membrane (Fig. 5a). The first

Table 1

Overview of the similarities and differences between cardiomyocyte dedifferentiation in co-culture and in vivo

	Normal	CHM	CAF	Co-culture
Myofibrillar loss	–	Perinuclear	Perinuclear	Random
Redistribution heterochromatin	–	+	+	+
Abberantly shaped mitochondria	–	+	+	+
Glycogen accumulation	–	++	++	–/+
Striated expression pattern titin	+	–	–	–
Striated expression pattern desmin	+	–	–	–
Expression pattern cardiotin	+	–	–	–
Expression α -smooth muscle actin	–	+	+	+

CHM=chronic hibernating myocardium (man); CAF=chronic atrial fibrillation.

obvious ultrastructural changes coincided with the beginning of cell spreading initiated after interaction with fibroblasts. Many of the Z-lines were disrupted and the sarcomeres started to lose their alignment. Consequently, the mitochondria started to lose their alignment too (Fig. 5b). Small patches of glycogen granules could be noted in some of the myocytes. A gradual depletion of contractile material with loss of myofibrillar organization and structure was accompanied by the occurrence of aberrantly shaped — small and elongated — but healthy looking mitochondria (Fig. 5b). The nuclear heterochromatin became uniformly dispersed throughout the nucleoplasm. After 2 weeks of co-culture, although retaining a more or less rectangular shape, myocytes were difficult to recognize as such. Only some remnants of myofibrils with small Z-line-like structures — mostly in the vicinity of the nucleus — enabled their identification (Fig. 5c). Nevertheless, these cells did not show obvious degenerative signs, such as mitochondrial swelling, extensive formation of lysosomes, or the loss of sarcolemmal integrity.

4. Discussion

Cardiomyocyte dedifferentiation as seen in chronic hibernation and several other cardiac pathologies, is a poorly understood form of cellular remodeling. It is clear that questions about initiation and progression of the dedifferentiation can only be assessed in model systems, and in this context cellular models are needed in particular. At present, animal models of hibernating myocardium are very scarce and to the best of our knowledge, no work has been reported to characterize cellular models of hibernation-like dedifferentiation. Although there has been considerable attention paid to spontaneous dedifferentiation events in adult rat cardiomyocytes *in vitro*, it became clear that this form of dedifferentiation is only partially comparable to the morphological changes *in vivo* [11–14]. For extensive research into the onset and progression of *in vivo* dedifferentiation, a cellular model mimicking these changes as much as possible, could be a valuable tool.

With this study, we show that by using a co-culture of adult rabbit cardiomyocytes and cardiac mesenchymal cells (mainly fibroblasts), we were able to induce typical morphological changes in the myocytes, which occur neither spontaneously in the same time frame, nor after application of FCM or fibroblast-grown cell inserts. With regard to the changes in protein expression and ultrastructure, ‘hibernating’ cardiomyocytes *in vivo* and these co-cultured cardiomyocytes showed a high degree of similarity (Table 1). The changes in the pattern of expression of the proteins titin, desmin, cardiotin, α -actinin and α -smooth muscle actin have been described in detail previously in myocardial tissue from patients with chronic hibernating myocardium [16] and in atrial tissue from goats with chronic atrial fibrillation [6]. In the present

study, the 9D10 epitope from titin displayed a normal cross-striated pattern in freshly isolated cardiomyocytes and in cultured myocytes prior to fibroblast interaction. After fibroblast interaction, however, this distribution pattern gradually disintegrated. In dedifferentiated co-cultured cardiomyocytes, the titin epitope appeared only sparsely and in a punctate fashion, as it was observed in severely dedifferentiated cardiomyocytes from chronic hibernating myocardium and chronic fibrillating atrium [6,16]. The sequence of changes of the titin pattern in the co-cultured cardiomyocytes was the reverse of that during embryogenesis. Indeed the punctate expression pattern could be observed in early stages of cardiac myofibrillogenesis of rabbit embryos [17]. In contrast, the titin-associated protein α -actinin could be found in a normal pattern in the remaining sarcomeres, just as it is the case in cardiomyocyte dedifferentiation *in vivo* [6]. During embryonic development desmin changes its organization in the heart from a filamentous into a cross-striated pattern. Thereafter, the epitope showed increased staining at the intercalated disk [17]. The co-cultured cardiomyocytes followed a contrary pathway: desmin was lost at the distal cell ends and gradually lost its normal cross-striated pattern. This change can also be observed in dedifferentiating cardiomyocytes *in vivo* during chronic hibernation or chronic atrial fibrillation, as well as in other pathologies such as dilated cardiomyopathy [6,18]. In spontaneously dedifferentiated adult rat cardiomyocytes a similar changed (filamentous) desmin pattern could be observed in quiescent myocytes in their early phase of spreading [19]. It can be hypothesized that desmin rapidly undergoes organizational alterations in response to changes in extracellular architecture and/or to changes in contractile activity. Indeed, adult rat cardiomyocytes gaining spontaneous contractions in long-term culture, partly regain periodic arrangement of desmin along the Z-line, but a filamentous network remains present [19].

In chronic hibernating myocardium [16] and chronic fibrillating atrium [6], re-expression of α -smooth muscle actin could be detected in some of the dedifferentiated cardiomyocytes. A similar diffuse staining pattern of this embryonic actin isotype was observed in some of the co-cultured cardiomyocytes in long-term culture. The heterogeneous response in re-expression of α -smooth muscle actin may be explained by different stages of myocyte dedifferentiation in the same culture. Indeed, cardiomyocytes that had still a rather well-developed sarcomeric apparatus were consistently negative for α -smooth muscle actin, whereas only those myocytes that had few sarcomeres left — probably representing a more dedifferentiated state — displayed a strong staining for this protein. In contrast to the situation of cultured adult rat cardiomyocytes [14], no structural organization of α -smooth muscle actin was seen. In our hands, α -smooth muscle actin only displayed diffuse staining, just as is the case in dedifferentiated cardiomyocytes *in vivo*. Perhaps

the spontaneous contractile activity of adult rat cardiac myocytes in long-term culture accounts for the structural organization of the protein.

Recent findings suggest that the disappearance of the normal longitudinally oriented cardiotin strands recognized by the R2G antibody, is one of the first changes that can be detected in a study of dedifferentiating cardiomyocytes in goats with chronic atrial fibrillation [20]. It was reported that the extent of cardiotin disappearance in human biopsies was coupled to the degree of structural alterations. Shorter filaments were noticed in the cells least affected, whereas the cardiotin pattern had completely disappeared in the severely affected cardiomyocytes. Our findings indicate that the normal longitudinally oriented cardiotin arrays could be observed only immediately after isolation. Soon after culturing, these arrays became shorter, to ultimately leave only some minor dots. These findings seem to confirm the changes *in vivo*; however, it should be emphasized that these changes — unlike the other structural alterations — occurred before fibroblast interaction, thus long before any other changes could be observed. Although the exact role of cardiotin is still obscure, we hypothesize that some dedifferentiation changes occur spontaneously (independent of fibroblast interaction) and very rapidly in cultured cardiomyocytes. Indeed the loss of its normal physical environment and contractile activity leaves the cell in a totally different mechanical and energetic state, which might be enough to trigger some characteristics of dedifferentiation [21,22]. It should also be stressed that, with the exception of cardiotin, the changes of all of the structural proteins examined, were consistently linked with cell spreading. Changes in the protein expression pattern of titin, desmin and α -smooth muscle actin were not seen before cell spreading occurred (after fibroblast interaction).

The observed ultrastructural changes in dedifferentiating co-cultured myocytes are in many ways also similar to those in dedifferentiated cardiomyocytes *in vivo*. Ultrastructural alterations such as myofibrilolysis, mitochondrial shape changes, and nuclear heterochromatin redistribution were all very like the changes reported in dedifferentiating cardiomyocytes in chronic hibernating myocardium and chronic fibrillating atria [1,6]. However, there were some differences as well: myolysis *in vivo* occurs perinuclear, whereas it occurred in a more random fashion in the co-cultured cardiomyocytes. The reason for this difference might be the different extracellular environment of the cells *in vivo* and *in vitro*, and therefore the different passive load (stretch). It has been shown that stretch is an important factor in maintaining myofibrillar organization, and therefore an altered three-dimensional load between *in vivo* and *in vitro* might account for the difference in preferential localization of the loss of myofibrils [21–23]. One of the most prominent changes in hibernating cardiomyocytes is the extensive glycogen accumulation. This typical change seemed to be barely

present in the co-cultured cardiomyocytes. Only some myocytes displayed minor glycogen accumulation, observed ultrastructurally as accumulation of small glycogen clusters. Again a mechanically and energetically different environment compared with the *in vivo* situation might account for this difference. Alternatively this finding might indicate that, unlike the structural changes, the metabolic changes (increased glycogen accumulation) depend on a persistent or repetitive decreased oxygen availability [24].

It is known that at least some of these ultrastructural changes and alterations in protein expression patterns also occur spontaneously, without fibroblast interaction. Indeed, Decker et al. [25] described that quiescent adult rabbit cardiomyocytes spread and showed myofibrillar disruption after 2 weeks in culture. However, these spontaneous ‘dedifferentiating’ rabbit cardiomyocytes changed in a somewhat different way from that which we observed. For example, these myocytes rapidly formed extended attachment plaques at their distal ends, which we did not observe in our setting. The ultrastructural appearance was also different from the morphology reported here. However, subtle differences in the experimental setup, such as serum and laminin concentrations, might be responsible for the different behaviour. The present study clearly indicates that the fibroblast interaction elicits morphological changes characteristic of dedifferentiation much earlier than would occur without it, indicating that co-culturing with fibroblasts may induce a different kinetic of dedifferentiation. When freshly isolated cardiomyocytes and fibroblast suspensions are seeded to a 2:1 ratio, the changes occurred even sooner (after 1–2 days) than in the current setting, where fibroblast proliferation had to be awaited (Dispersyn, unpublished data). Even though it is beyond the scope of this paper to further characterize the cardiomyocyte — fibroblast interaction, it is obvious from the present findings that factors secreted by fibroblasts are not solely responsible for the dedifferentiation. Indeed, when considering d5 or d6 cultures, cardiomyocyte dedifferentiation could only be seen in cardiomyocytes in close contact with fibroblasts and could not be induced by application of FCM or fibroblast-grown cell inserts. Nevertheless, it cannot be ruled out that locally produced or activated paracrine factors in combination with close cell–cell interactions are essential in this setting.

With their co-culture model of adult rat cardiac myocytes and epicardial mesenchymal cells (non-fibroblasts), Eid et al. [15] showed that co-cultured cells can influence cardiomyocyte morphology. But in contrast to our study, Eid et al. noted increased differentiation of the rat myocytes in close apposition with the co-cultured counterparts. A possible explanation for these divergent findings is that the co-cultured cells in the aforementioned work induced large amplitude contractions in the myocytes — a behaviour that did not occur in our setting. Active loading of cultured myocytes has been shown to positively influence the degree of differentiation of cultured myocytes

[22,26]. The increased differentiation could not be induced when fibroblasts rather than epicardial mesothelial cells were used, but it was not stated whether these cells had a 'dedifferentiating' effect. Further research is presently ongoing to explore the intercellular interaction and possibly identify important triggers or co-triggers of hibernation-like dedifferentiation in our co-culture model.

In conclusion, we have presented an *in vitro* model of adult rabbit cardiomyocytes in which dedifferentiation similar to *in vivo* dedifferentiation can be induced. This model consists of co-culturing adult rabbit cardiomyocytes with cardiac fibroblasts and mimics the most important structural changes of dedifferentiating cardiomyocytes *in vivo*, and may therefore be an important tool for further investigating possible triggers for and the progression of dedifferentiation *in vivo*. We suggest that most of the structural changes in dedifferentiating cardiomyocytes *in vivo* are not directly dependent on changes in oxygen availability. Further research is currently ongoing to characterize the triggers elicited by the fibroblasts on the cardiomyocytes in co-culture.

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