C_2C_{12} murine myoblasts as a model of skeletal muscle development: morpho-functional characterization

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In this study, the differentiation of C2C12 cells, a primary line of murine myoblasts, was investigated by a multiple technical approach. Undifferentiated cells, and those at intermediate and final differentiation times, were studied at the reverted microscope, by conventional and confocal immunofluorescence, and by transmission and scanning electron microscopy. The general monolayer architecture changed during differentiation from fusiform or star-shaped cells to elongated confluent cells, finally originating long, multinucleated myotubes. Sarcomeric actin and myosin are present also in undifferentiated myoblasts, but progressively acquire a structured pattern up to the appearance of sarcomeres and myofibrils at about 5 days after differentiation induction. Myotubes show a particular positivity for actin and myosin, and M-cadherin, an adhesion molecule characteristic, as known, of satellite cells, also seems to be involved in their assembling. Rare apoptotic patterns, as evidenced by the TUNEL technique, appear during myoblast maturation.

Key words: C_2C_{12} myoblasts, differentiation, actin, myosin, ultrastructure

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Mammalian skeletal myogenesis is a complex phenomenon, taking place from the first weeks of embryonic development. Totipotential mesenchymal cells provide a population of mononucleated fusiform cells, named myoblasts and known to be the precursors of contractile muscle cells. Myoblasts progressively fuse to form plurinucleate syncytia, the myotubes (Lawson and Purslaw 2000), which furtherly differentiate to acquire the final morpho-functional features of the muscle cells (Ordahl 1993; Carozzi et al. 2000).

Under the control of regulatory proteins and as a consequence of neural and/or hormonal actions, the skeletal muscle fibre enlarges and displays contractile structures and functions (Ogilvie et al. 2000).

MRFs (myogenic regulatory factors) are proteins exclusively expressed in cells committed to the myogenic lineage which alternately appear and disappear during differentiation. Myo-D, Myf-5, MRF-4 and myogenin are, to date, the best characterized, and become active in a space and time-correlated manner (Krempler and Breing 1999; Delgado et al. 2003).

Myo-D seems to activate the transcription program of muscle-specific genes, but also permanently arrests the cell cycle by inhibiting transcription factors (Kataoka et al. 2003). It is known that Myo-D and Myf-5 regulate paraxial muscles (migratory and limb muscles, diaphragm, surface trunk muscle, respiratory and deep trunk muscles) (Pownall and Emerson 1992a,b). Both genes activate myogenin, which is associated with final muscle differentiation processes (Thayer et al. 1989). MRF-4 (Rhodes and Konieczny 1989) may play a role in the regulation of muscle fiber phenotype in postnatal life, especially in maintaining the slow phenotype (Walthers et al. 2000).

MRFs induce the expression of other muscle specific proteins, such as alpha-actin, myosin heavy and light chains, tropomyosin, troponin-C and troponin-I.

Most MRFs, when overexpressed in fibroblasts, nerve cells, liver cells and adipocytes, induce these cells, of mesodermal origin, to become muscle cells. They thus have a definite myogenic activity (Dedieu et al. 2002).

A number of hormones have been also demonstrated to affect muscle development and function. Myofibers have insulin receptors, and the effects of this hormone have been widely reported (Goel and Dey 2002). Resting muscle indeed needs insulin to increase in size (Conejo and Lorenzo 2001): when the hormone is secreted, excess nutrients can be stored in muscle; if insulin concentration is low, protein stores are progressively mobilized and muscle atrophy may ensue.

Thyroid hormones are required for myofiber maturation (Merkulova et al. 2000); differently, autocrine growth hormone production has been recently reported to inhibit differentiation (Segard et al. 2003).

Androgens and estrogens, whose receptors are present in skeletal myofibers, seem to stimulate muscle protein synthesis (Lee et al. 2003) and glucocorticoid hormone stimulates mitochondrial biogenesis (Weber et al. 2002).

Aside from muscle differentiation, a number of residual myoblasts escape skeletal muscle development and persist in adult muscles as "satellite cells", localized between the sarcolemma and the basal membrane of muscle fibers (Cooper et al. 1999). In particular conditions, such as denervation, physical exercise stress, muscle damage, others, they proliferate, differentiate and fuse to form fiber cells for the repair of damaged muscle tissue (Morgan and Partridge 2003). Satellite cells mediate postnatal muscle growth, and their population decreases with age. In the quiescent stage, they appear rounded and mononucleated, and do not express relevant amounts of myogenic proteins.

C2C12 are murine myoblast cells derived from satellite cells, whose behavior corresponds to that of progenitor lineage. These cells are a subclone of C2 myoblasts (Yaffe and Saxel 1977) which spontaneously differentiate in culture after serum removal (Blau et al. 1983). Cycling myoblasts are comparable to activated satellite cells in muscle fibers, while the cells referred to as "resting" (Yoshida et al. 1998), correspond to the quiescent satellite cells.

In this study, we characterized the C2C12 myoblast cells during myogenic differentiation. The time-course of their general behavior was followed by frequent reverted microscope observations; actin and myosin arrangement was monitored by immunofluorescence; surface cell features were described by scanning electron microscopy; and structural details of inner cells were analyzed by transmission electron microscopy. M-cadherin was also investigated and, finally the role of programmed cell death during differentiation was considered.

Materials and Methods

Cell Culture

C2C12 mouse adherent myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum, 2mM glutamine, 1% antibiotics, 0.5% antimycoplasm and 25mM Hepes, pH 7.5. The cell line was maintained in a 5% CO2 atmosphere at 37°C and cell viability was assessed by the Trypan Blue exclusion test (Luchetti et al. 2003). To induce myogenic differentiation, when about 80% cell confluence was attained, the medium containing 10% fetal calf serum was substituted with 1% fetal calf serum (Lattanzi et al. 2000; Columbaro et al. 2001; Faenza et al. 2003).

The cells were observed at critical time intervals, i.e. at the undifferentiated stage and at 3, 5, 7, 10 days of differentiation, with a Nikon Eclipse TE 2000-S reverted microscope (RM) and photographed with a digital DN 100 Nikon system.

Immunofluorescence (actin, myosin, M-cadherin, TUNEL)

Immunofluorescence (IF) techniques were carried out directly in dishes containing a cover slide, where cells had been seeded and became adherent.

IF analysis was performed on the undifferentiated cells and at all times of the differentiation course. Cells were rinsed with 0.1M PBS, pH 7.4, fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature (R.T.), again washed with PBS and stored at 4°C until all time points were collected.

Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes at R.T., rinsed with PBS and afterwards prepared for immunocytochemical or cytochemical techniques for the detection of the markers as described below.

For labelling of muscle actin (Luchetti et al. 2002), cells were treated with 5% normal horse serum (D.B.A.) and 2% bovine serum albumin (BSA; Sigma) in PBS for 30 minutes at R.T. and then incubated with a mouse antibody against α-sarcomeric actin (Sigma; 1:100 in PBS) overnight at 4°C. After rinsing in PBS, the specimens were incubated with a FITC-conjugated horse anti-mouse secondary antibody (D.B.A., Vector; 1:50 in PBS) for 1 hour at R.T., stained with propidium iodide (PI), 1 μg/ml in PBS for 5 minutes and mounted with an antifading medium.

A cytochemical technique was used for the visualization of actin filaments. Cells were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes at R.T. and rinsed with PBS. Successively, they were treated with FITC-conjugated-phalloidin (Sigma), 25 μg/mL for 60 minutes at R.T. and counterstained with with PI, 1 μg/mL in PBS for 5 minutes.

For the detection of myosin, cells were treated with 5% normal goat serum (D.B.A.) and 2% BSA in PBS for 30 minutes at R.T. and then the cells were incubated with a rabbit anti-skeletal myosin (Sigma, M7523; 1:50 in PBS) overnight at 4°C. This antibody recognizes both light and heavy chains. After rinsing in PBS, cells were incubated with a FITC-conjugated anti-rabbit (D.B.A., Vector, 1:50 in PBS) for 1 hour at R.T. and stained with PI, 1 μg/mL in PBS for 5 minutes.

The expression of M-cadherin was investigated with an immunohistochemical technique. Cells were treated with 5% normal goat serum (D.B.A.) and 2% BSA in PBS for 30 minutes at R.T. and then incubated with a rabbit anti-M-cadherin antibody (Santa Cruz; 1:200 in PBS) overnight at 4°C. After washing in PBS, cells were incubated with a FITC-conjugated goat anti-rabbit secondary antibody (D.B.A., Vector; 1:50 in PBS) for 1 hour at R.T. and stained with PI, 1°g/ml in PBS, for 5 minutes.

For TUNEL staining, cells were permeabilized with 0.2% Triton X-100 as previously described. Apoptotic cells were detected by DNA fragmentation using the TUNEL method, which specifically labels the 3'-hydroxyl termini of DNA strand breaks (Gavrieli et al. 1992). For the TUNEL procedure, all reagents were part of a kit (Apoptag Plus, D.B.A., Oncor) which utilizes a digoxigenin-conjugated dUTP, followed by a FITC-conjugated anti-digoxigenin antibody. All the procedures were performed according to the manufacturer's instructions and, finally, cells were stained with PI, 1 μg/mL in PBS for 5 minutes at R.T. and mounted with an antifading medium.

All specimens were observed and photographed with a fluorescence microscope (Vanox Olympus MI, Italy): for viewing both FITC and PI fluorescence, a combination of BP 490 and EY 455 excitation filters were used. Slides for M-cadherin analysis and TUNEL detection were observed by means of an Olympus FV300 laser-scanning microscope (Olympus MI, Italy).

Scanning Electron Microscopy (SEM)

Cells were directly cultured on cover slides inside Petri dishes. After careful washing with phosphate buffer, monolayers were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 hour quickly washed and post-fixed with 1% OsO_4 in the same buffer for 1 hour. After alcohol dehydration, they were critical point dried, gold sputtered (Luchetti et al. 2003) and observed with a Philips 515 scanning electron microscope.

Transmission Electron Microscopy (TEM)

C2C12 cells growing in flasks were washed and immediately fixed with 2.5% glutaraldeyde in 0.1M phosphate buffer for 15 min, gently scraped and centrifuged at 1200 rpm. Pellets were additionally fixed for 1 hour, alcohol dehydrated, and embedded in araldite (Falcieri et al. 2003). This procedure allowed a good maintenance of the cell shape and prevented direct TEM of cell monolayers which, even if possible, provides limited information and is technically difficult to carry out. Thin sectioning was preceded by the analysis of toluidine blue-stained semithin sections (Falcieri et al., 2000b) which allowed an overall specimen view. Thin sections were stained with uranyl acetate and lead citrate and analysed with a Philips CM10 electron microscope.

Results

Monolayer study was carried out at the undifferentiated stage, i.e. 1-2 days after plating, and at 3, 5, 7 and 10 days after differentiation induction, the undifferentiated condition, the intermediate maturation period and the final differentiation time being the most critical experimental steps to which the cell images are referred.

Careful cell monitoring was performed at the RM

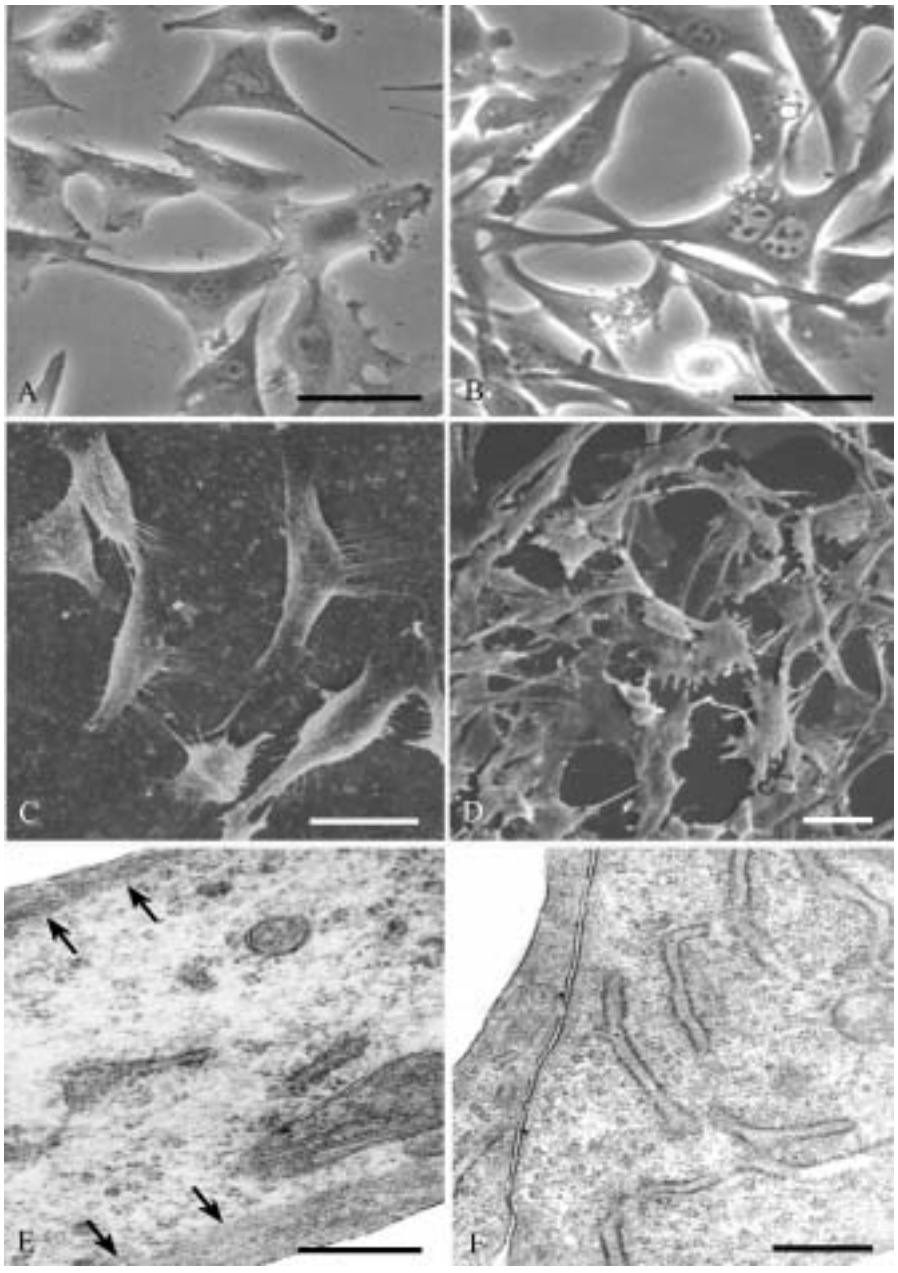

Figure 1. Undifferentiated C2C12 cells monolayers at RM (A,B), SEM (C,D) and TEM (E,F): they appear star-shaped or fusiform (A,B) and closely adherent to the substrate (C). Mitoses (B), with progressive cell rounding and detachment (D) are clearly identifiable. Stress fibers (→) also appear, as bundles of thin filaments in subsarcolemmal areas (E); an abundant, slightly dilated, rough endoplasmic reticulum, is also present (F). A,B, bar = 25 µm; C,D, bar = 23 µm; E, bar = 1 µm; F, bar = 0.5 µm.

to assess the effectiveness of the differentiation induction and to choose the experimental points to investigate. IF was utilized to identify actin and myosin arrangement and localization, as well as M-cadherin-positive cells, which were better characterized by confocal microscopy. IF was also performed to identify DNA fragmentation by the TUNEL reaction. SEM allowed description of monolayer surfaces and cell interactions during differentiation. TEM provided a detailed cell insight, with particular regard to appearance and organization of contractile structures and to events correlated with myotube formation.

Undifferentiated cells, when observed at the RM, show a 20-80 µm size, appear flat, not confluent and closely adherent to the substrate. They are star-shaped or fusiform, rigorously showing one central nucleus, with numerous nucleoli (Figure 1A). A number of cells, in different mitotic phases, can also be found (Figure 1B).

SEM reveals cell phillopodia and large intercellular free spaces (Figure 1C). At lower magnification, in cell contact areas, a tendency to cellular overlapping appears, but cell rounding and detachment

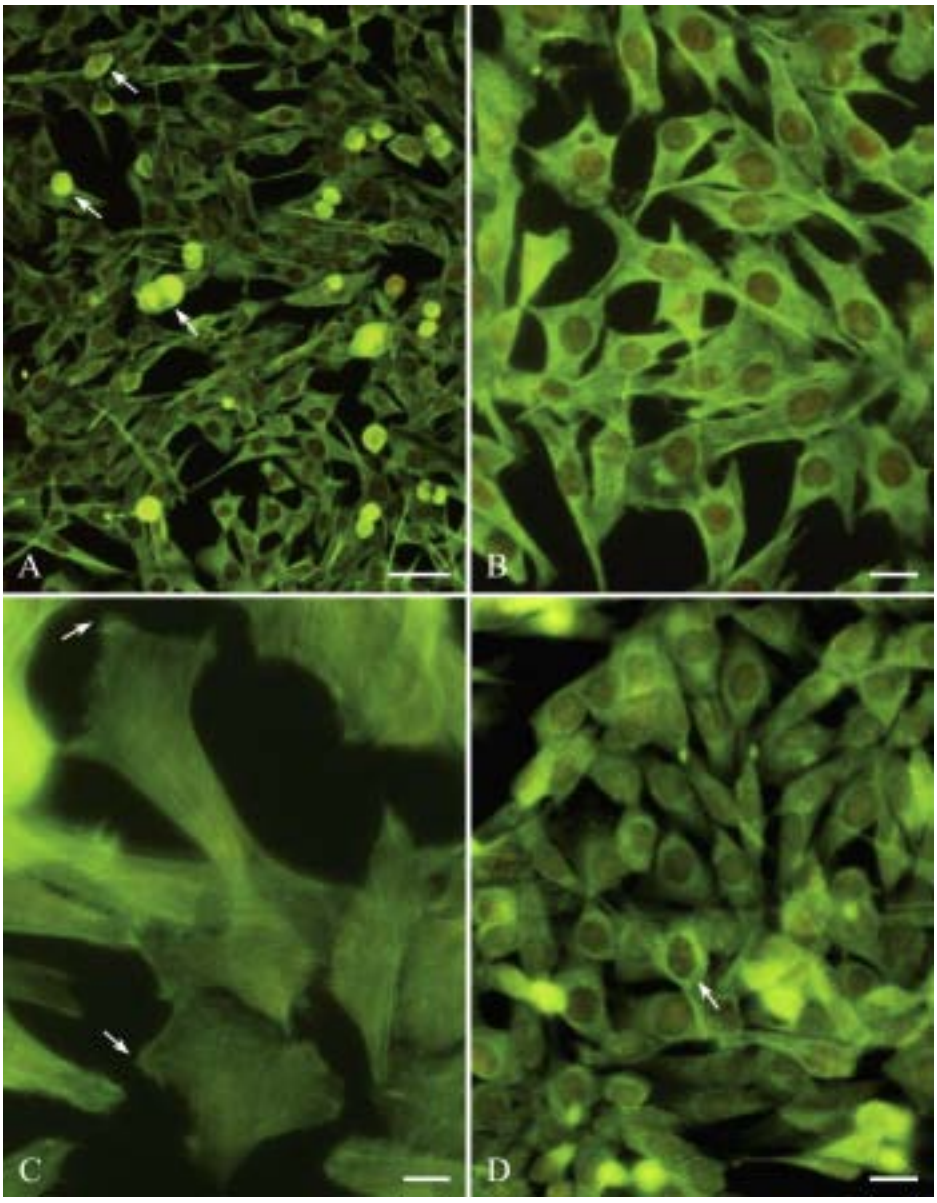

Figure 2. IF of undifferentiated C2C12 cells labelled with anti-actin antibody (A,B), phalloidin (C) and anti-myosin antibody (D): mitoses are clearly identifiable (A, →) and a diffuse actin distribution (A,B), more evident in subsarcolemmal regions (C, →), appear. Myosin shows an initial localization in perinuclear areas (D, →). A, bar= 50 µm; B,D, bar = 20 µm; C, bar = 10 µm.

seem to be exclusively associated to mitotic cell rearrangement (Figure 1D).

Bundles of stress-fibers (Langanger et al. 1984), in sub-sarcolemmal domains also appear at TEM observation (Figure 1E) and rough endoplasmic reticulum, slightly dilated, is still widely identifiable at this stage (Figure 1F). At the undifferentiated stage, actin appears diffusely distributed throughout the cytoplasm (Figure 2A,B). In fact, the antibody recognizes α-sarcomeric actin and thus also binds to cardiac actin, which is largely expressed at this stage (Bains et al. 1984). PI nuclear staining very rarely shows apoptotic or necrotic patterns, while mitoses can be promptly identified in yellow because of the red-green simultaneous fluorescence (Figure 2A, arrows). Areas characterized by a more concentrated assembly of actin bundles appear, after phalloidin staining, at the cell periphery, probably correlated to stress fiber presence (Figure 2C, arrows) (Coghill et al. 2003).

Myosin is also widely expressed: however, it shows a more homogeneous localization and, differently from actin, seems more concentrated around the nucleus (Figure 2D, arrow).

At the intermediate differentiation stage, cells tend to acquire a mainly elongated shape, intercellular spaces progressively recede and initial confluence patterns can be observed throughout the monolayer, both at RM (Figure 3A) and SEM (Figure 3B).

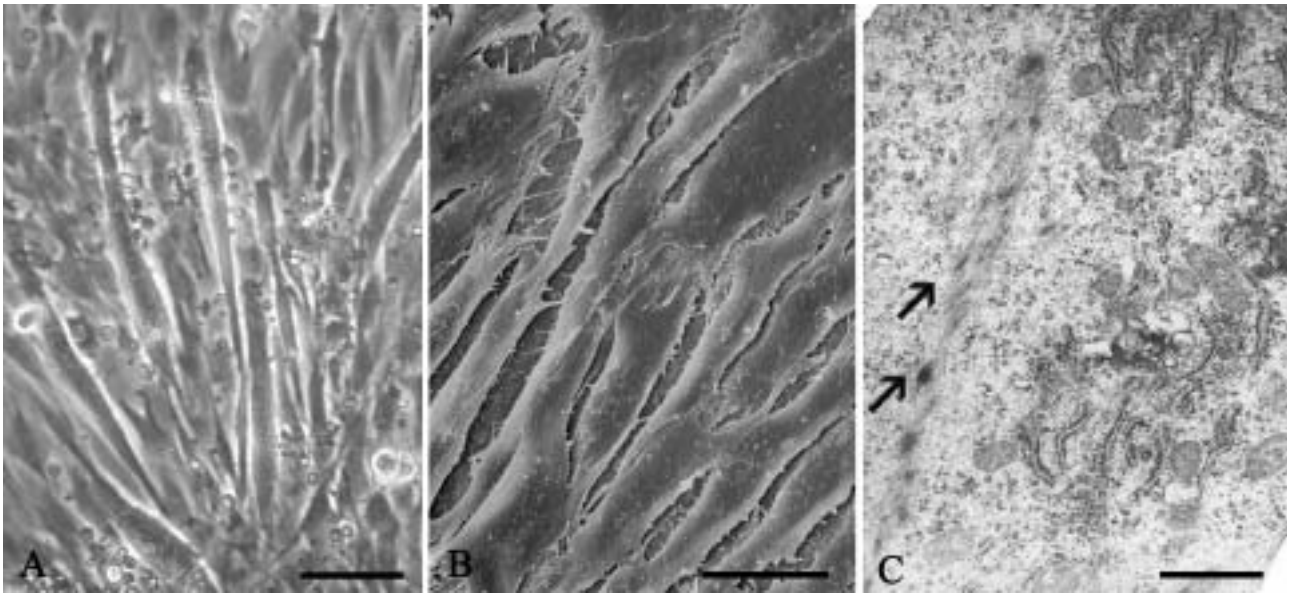

Figure 3. Intermediate differentiation stage: C2C12 cells become more elongated and, progressively, confluent, both at RM (A) and SEM (B). Promyofibrils and z-bodies (→) appear at TEM (C). A, bar = 50 µm; B, bar = 20 µm; C, bar = 0.5 µm.

Ultrastructural analysis of C2C12 cell cytoplasm reveals an initial myofibril arrangement. Progressive spatial organization of thin filaments occurs and Z-bodies, known as the primitive actin anchoration structures (Sanger et al. 2002), can be revealed (Figure 3C). In cytoplasm, mitochondria and rough endoplasmic reticulum are observable.

At high cell differentiation conditions, myotubes appear. They are 100-600 µm long, 30-50 µm thick, fusiform structures (Figure 4A), frequently also elongated in three-four directions (Figure 4B). They show numerous (even more than 20) nuclei, centrally localized and clumped or distributed all along their extension, as PI staining shows in detail (Figure 4C).

TEM confirms C2C12 maturation. Within cytoplasmic thin filaments, thicker ones appear and both organize to form sarcomeres and myofibrils. Cross sections show in detail the relationships between thin and thick filaments, which appear closely comparable to those typical of adult skeletal muscle (Figure 4D, E, F).

C2C12 myotubes appear strongly actin-positive, and are promptly recognized from the surrounding single cell monolayer (Figure 5A, B, C). Curiously, some of them occasionally show a sort of sarcolemmal blebbing, which may be correlated, also considering electrophysiological membrane patterns (data not shown) to initial contractions (Figure 5D, arrows). Myosin, similarly to actin, seems to label myotubes much more avidly than it labels mononucleated cells (Figure 5E), and is again well recognizable in mononucleated cells in perinuclear areas (Figure 5F, arrows).

Myotube formation has been also investigated by characterizing M-cadherin behavior. This protein, involved in adhesion mechanisms (Donalies et al. 1991; Kaufmann et al. 1999a), occasionally appears in C2C12 myoblasts. Rare positive, curiously elongated, cells can be found indeed in the early differentiation condition (Figure 6A). To better investigate this phenomenon, confocal microscopy was used, which showed at intermediate differentiation times a relevant M-cadherin positivity in plurinucleated, myotube-forming, cells (Figure 6B).

Apoptosis can also be identified in middle and final maturation conditions. DNA fragmentation appears indeed "in situ" after TUNEL reaction at confocal microscopy (Figure 6C) and at the ultrastructural level (data not shown).

Giant cells, strongly positive for actin and myosin, are a peculiarity occurring all along C2C12 differentiation (Figure 6D,E,F,G).

Discussion

This morphological approach to the study of C2C12 myoblasts maturation in vitro demonstrates the effectiveness of the culture technique. In fact,

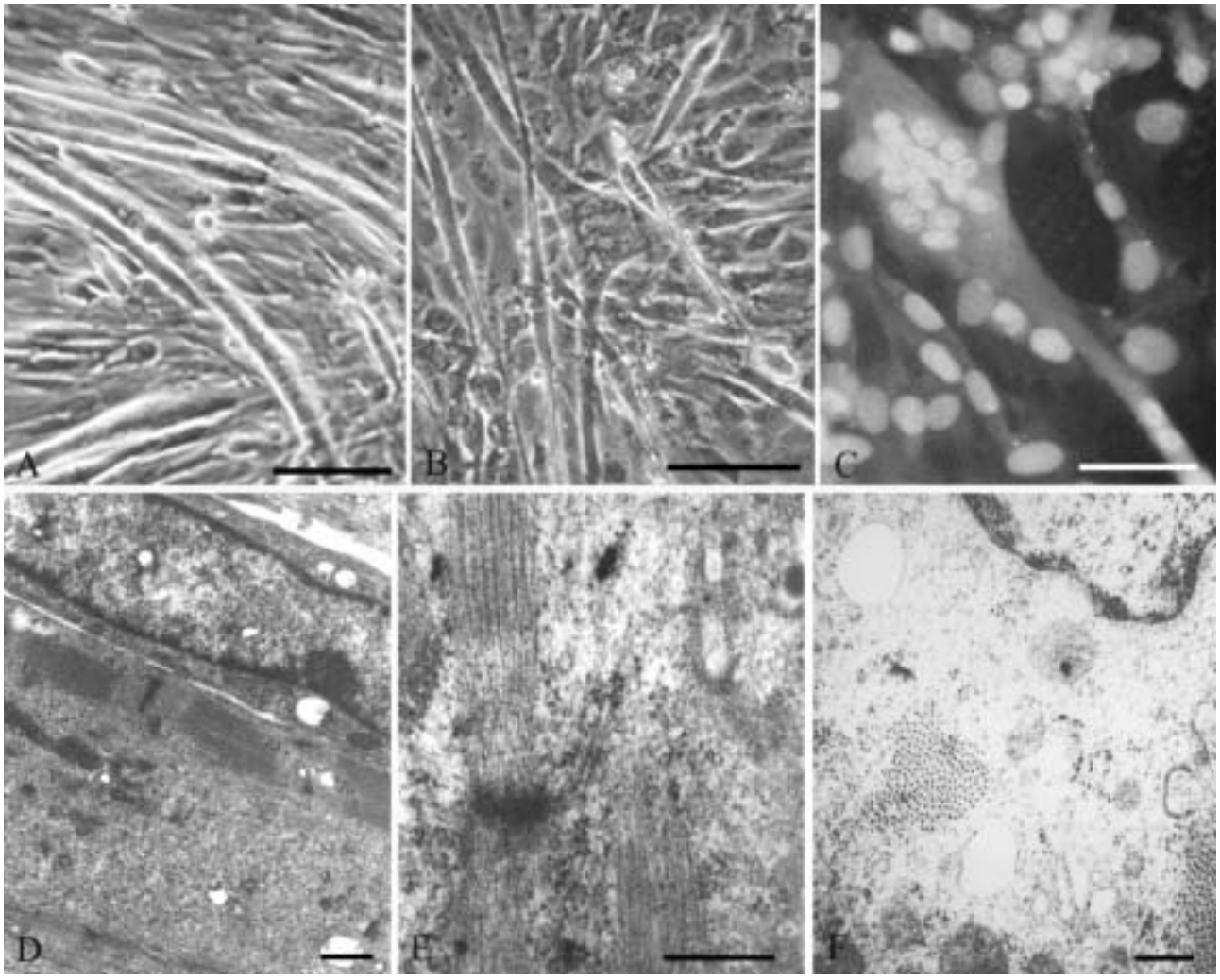

Figure 4. In high differentiation phases, elongated (A) or y-shaped (B) myotubes appear at RM (A,B) and IF (C); after PI staining, the numerous nuclei are clearly visible (C). Thin and thick filaments, organized in sarcomeres and myofibrils (D,E) appear. The reciprocal space organization is visible in cross section (F). A,B, bar = 100 µm; C, bar = 50 µm; D, bar = 0.5 µm; E,F, bar = 0.2 µm.

starting from an apparently homogeneous undifferentiated myogenic line, a population, numerically around 50% of cells, differentiate (Yoshida et al. 1998). The remaining cells presumably remain in a quiescent myoblastic state.

In the undifferentiated condition, the C2C12 myoblasts are flat, fusiform or star-shaped mononucleated cells. They express sarcomeric and filamentous actin, mainly localized at the cell periphery and focal contacts; they also appear positive for skeletal myosin (Kataoka et al. 2003). Although myosin is one of the main components of the contractile apparatus to become well structured in late differentiation, its presence in undifferentiated cells is a well-documented phenomenon both in myoblasts (Salamon et al. 2003) and in other cell lines (Ezzel et al. 1992).

Myogenic differentiation is recognizable by a progressive change in cell shape and monolayer organization. The cells become elongated, and structures involved in contraction progressively assemble. Bundles of thin filaments, closely associated to Z bodies, known as the primordial actin anchoration sites (Sanger et al. 2002), can be revealed.

The presence of elongated, M-cadherin-positive cells is an intriguing observation. It is indeed well known that this adhesion molecule, which mediates cell-cell adhesion relationships (Kaufmann et al. 1999a), can be considered a satellite cell marker (Cooper et al. 1999). Moreover, the appearance of

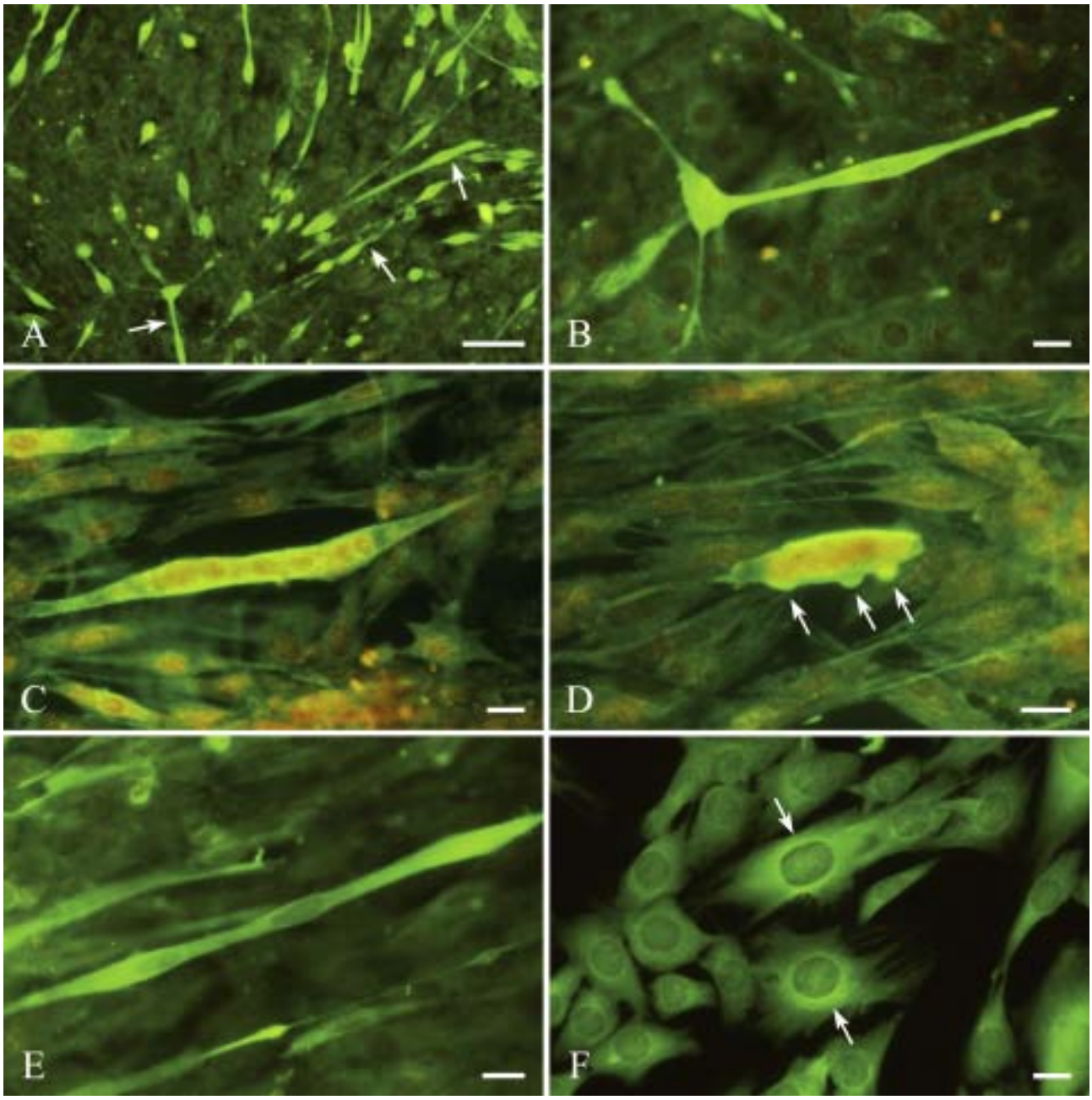

Figure 5. High differentiation times: myotubes are intensely positive for actin (A, →),B,C,D) and for myosin (E), more than are underlying mononucleated cells. Surface blebs (D, →) frequently appear. Myosin staining, in mononucleated cells (F, →) appear, again, mainly in the perinuclear region.

this molecule in assembling myotubes, as shown by confocal microscopy, suggests its correlation with myotube formation. On the other hand, neither IF nor even ultrastructural analysis have ever permitted precise characterization of the very early phenomenon of muscular syncytia formation. It is, however, reasonable that an adhesion molecule, such as M-cadherin, has a role in their assembling. Therefore, its presence in plurinucleated myotube-forming cells suggests its possible role also in muscular syncythia biogenesis as previously reported in other experimental models (Kaufmann et al. 1999b).

Myotubes appear from 5 days of differentiation in our system. They are elongated (100-600 μm), thick (30-50 μm) syncythia with more than 20 nuclei and occasionally show a Y-shape (Figure 4B, 5B). They show a certain rigidity, frequently deter-

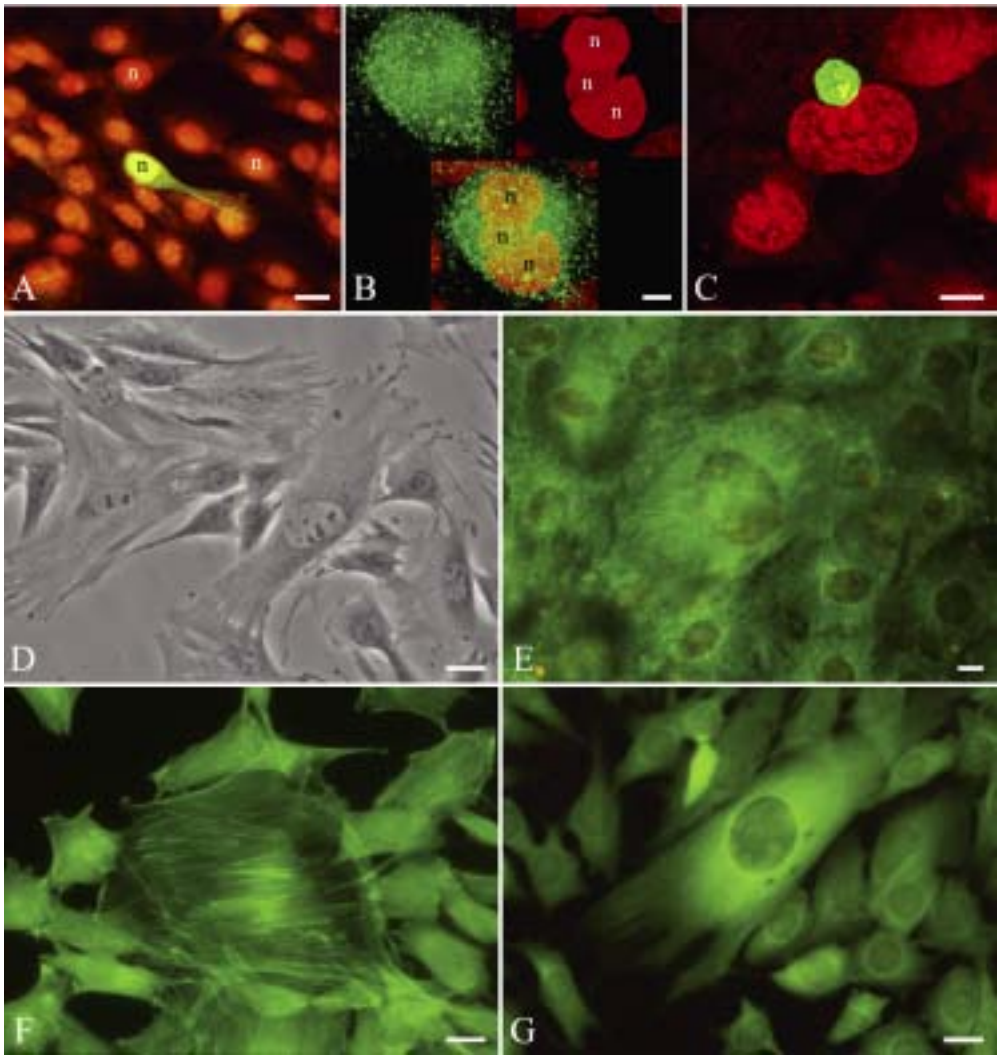

Figure 6. M-cadherin localization (A,B), TUNEL reaction (C) and giant cells (D,E,F,G) at undifferentiated (A) and intermediate (B,C,D,E,F,G) stages of differentiation. M-cadherin appears in elongated cells (A) and in forming myotubes (B, confocal microscopy: on the left above, M-cadherin-immunopositivity in green; on the right above, PI staining in red; below, merging of the two signals shows M-cadherin-immunopositivity in green and PI staining in red; n= nuclei). Apoptotic nuclei appear during differentiation times (C, confocal microscopy of TUNEL assay: merging of the two signals shows TUNEL-positivity in green and PI staining in red). Giant cells appear at RM (D) and by means of IF, with anti-actin (E), phalloidin (F) and anti-myosin (G) staining. A,C,D,F,G, bar=20 µm; B,E, bar=10 µm.

mining their detachment from the monolayer.

Actin and myosin, when monitored by fluorescence methods, appear much more concentrated in the myotubes than in mononuclear cells. Nevertheless, sarcomeric and myofibrillar assembling does not seem necessarily correlated to cell fusion. Mature contractile structures appear, indeed, in mononuclear cells, and, differently, large myotubes without myofibrils but containing masses of disorderly clumped filaments, have been described (Berendse et al. 2003).

Curious features of membrane ruffling or blebbing (Figure 5D) can be also observed at final differentiation times: this membrane behavior seems suggestive of primordial cell contractions.

In the absence of innervation, this hypothesis must be considered still speculative (Formigli et al. 2002). Nevertheless, monolayer movements can be occasionally observed at the RM and a relevant increase in Ca2+ channel number is revealed from electrophysiological membrane patterns (data not shown)

Substrates and structures for potential muscular contraction seem also to be present in our model.

Apoptosis has been widely described in differentiating systems (Luchetti et al.1998; Falcieri et al. 2000a; Luchetti et al. 2002; Burattini et al. 2003; Zamai et al. 2004) where it seems to balance cell proliferation during maturative progression. In particular stimulating conditions (Ceruti et al. 2000; Querfurth et al. 2001; Shiokawa et al. 2002), it has also been observed in muscle cell cultures. In our C2C12 model, it is scarcely, but consistently present even in reproducibly optimal culture and differ-

entiation conditions, and so may be considered as an intrinsic cell line behaviour rather than the consequence of inadequate experimental conditions (van den Ejinde et al. 2001). Interestingly, and in contrast with our predictions, it affects both early and late differentiated cells (apparently not myotubes), suggesting a certain C2C12 apoptotic commitment. Further experiments are necessary to better characterize the apoptotic phenomen and to highlight its possible role in pathology.

Giant cells represent a very particular feature, well identifiable at RM and after IF. They show an isodiametric, sometimes rounding, profile with a very large, undoubtedly polyploid, nucleus. They appear as actin- and myosin-positive, thus revealing morpho-functional features typical of differentiating muscle cells. It has been reported (Hieter and Griffiths 1999) that polyploidy is a naturally-occurring phenomenon which accompanies the differentiation process. Little is known about it, even if it is generally accepted that it could play roles in gene amplification, genome restructuring, chromosome interactions, and cell longevity (Hieter and Griffiths 1999; Leitch 2000). In our system, giant cell presence could be, in some way, correlated to initial myotube formation or to phenomena of myotube dedifferentiation, as has also been recently described (McGann et al. 2001). A technical approach aimed at myotube isolation is in progress in order to study them by means of flow cytometry and biochemical techniques, also in relationship with giant cells.

To conclude, our data demonstrate by a multiple technical approach the effectiveness of this differentiation system, which progressively transforms undifferentiated, fusiform or star-shaped myoblasts, into long multinucleated myotubes, which show the majority of contractile muscle cell features.

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