Muscle Differentiation of Normal and Double-Muscled Bovine Foetal Myoblasts in Primary Culture

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Abstract

Bovine muscle differentiation has been mainly studied *in vim.* The comparison during foetal growth of two genetic types; which display muscle hypertrophy. ;ind normal, have shown a delay in the evolution of the different myosin heavy chain isoforns in double-muscled compared tu normal foetuses. To investigate these observed differences we studied the growth in primary cultures of myoblasts taken from double-muscled and normal foetuses at 110 days posi-conception. These cultures were analysed at 2, 4, 6, 8, 12 and 14 days. The cell proliferation phase was monitored by means of corresponding marker antibodies, and the differentiation phase was characterised using a broad set of criteria. The total myofibrillar proteins were extracted and assayed. The proteins considered as early markers of muscle differentiation, such as desmin and connectin, were identified using antibodies. The different myosin heavy chain isoforms: foetal, alpha cardiac, slow and fast, were identified immimohistochemically and by immunoblotting. Creatine phosphokinase activity, another marker of muscle differentiation, was assayed at the different stages. The findings confirm the delayed differentiation of double-muse led foetuses and show this to be ihe consequence of a more intense cell proliferation phase. Key ttords: myoblast, bovine, muscle hypertrophy, myosin.

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The study of muscle differentiation in bovine is of special interest since the tenderness of the meat depends in par! on the composition of different muscle fibre types [20]. As in other species, the contractile differentiation of the muscle fibres involves two successive generations of cells [14, 18], However, in bovine, unlike other species, differentiation is well advanced at birth, showing that the foetal phase is an important period for muscle development. Double-muse led bovine (DM), which bear a gene for muscle hypertrophy, make a particularly interesting study model. These adult animals display strong muscle hypertrophy and their meat is very tender (for review see King and Menissier, [10]). At birth, their muscles comprise more large size fibres, with a higher proportion of IIB fibres (fast glycolytic) and a more glycolytic metabolism than those of normal bovine [1,9,13]. An in vivo study of ihe evolution of ihe different myosin heavy chain isoforms during the growth of DM foetuses compared with normal ones (N) showed a delayed muscle differentiation [15]. This delay, which affected both first and second generation muscle cells, was more marked during the first two thirds of the gestation period. Quinnetal. [17] suggested ihat the cell multiplication phase could be longer in the double-muscled foetuses and regulated by a myogenic factor produced by the fihroblasis in greater quantities in culsrds.

To investigate this *in vivo* delayed differentiation further we studied the muscle proliferation and differentiation phases in primary cultures of myoblasts from DM and N foetuses. The cells were studied at 2,4,6, 8, 10, 12and 14 days culture. The proliferation phase was analysed by monitoring the time course of the number of nuclei and rnononucleate cells, and by identifying various proteins characteristic of cell proliferation by means of antibodies. The differentiation phase was studied by the analyse of various markers of muscle differentiation; percentage of fusion, creatine phosphokinase aclivity, total myofibriliar proteins, desmin and connectin. Lastly, different myosin heavy chain isoforms; slow, fast, foetal and alpha cardiac, were identified itiummochemicaHy.

Material and methods

*Muscle samph'**

Double-muscled embryos of strain INRA95 [12] were transplanted into Charolais/Salers crossbreed heifers of a mean age of two years. Normal foetuses were produced by artificial insemination. Foetuses were taken just after

slaughter of the mother, at gestational ages of 110 days (tiillgestarional period for cattle is 270 days). At this stage previous studies have shown that the first and the second generation were present in the bovine muscle [18]. Four foetuses of the two types were obtained. Samples of *Longissimus thoracis* (LT) muscle were taken in sterile conditions and put in culture.

Ceil culture

Cell cultures were performed according to the prolocol of Buckingham etal. [5], modified by Beclietetal. [3]. I.T muscle samples were cut in small pieces in Ham's modified Eagle's medium (HMKM, Gibco URL) containing antibiotics : fungizone 2.5 ng/ml, penicillin 63)ig/ml. streptomycin 140 ug/ml, geniamycin 5 ug/ml. Muscle cells were dissociated by the addition of 0.25% trypsin (wt/vol) for 3 x 20 min at 20ªC. Trypsin was removed by centrifugation of the cells al 900 g for 10 min. Pelleted cells were then resuspended in Dubelco's modified Eagle's medium (DMEM, Gibco BRL) containing glucose 4.5 g/1, antibiotics described before and 10"u PCS. Cells were counted in an hemacytometer and plated onto Nunc 60 mm tissue culture dishes, without precoating, at a density of 10^5 cells/ml. Cultures were incubated at 37°C in a 5% COi atmosphere. Cells were led fresh medium 24 h after plating.

Histochemistry

Cells' nuclei were stained with Giemsa G250 colorant. Cells were washed with PBS and fixed with 4 ml of PBS/methanol (v/v)for 2 *x 2* min., then in 4 ml PBS/melhanol (v/2v). The coloration was done by incubation in Giemsa G250 0.04% in methanol for 2 min., then 3 volumes of distilled water were added and after 2 min cells were rinsed under water. After coloration the number of nuclei and cells were counted on 10 in randomly chosen fields. The percentage of fusion was determined as the percentage of nuclei incorporated into myotubes to the total nuclei number.

Antibodies

Four monoclonal antibodies (MAB. Biocytex Marseille, France) specific to different forms of myosins were used. The MAB S, F88 8H8. specific to slow MHC (MUC 1], was obtained from adult human auricle. The MAB R, I'l 13 15F4, specific for fast MHC (MHC 2a and 2b) was prepared from aduli myosin of rabbit Tibialis. The MAB F WHS obtained from a myosin of a 22-week-old bovine foetus (foetal MHC'}, anil the MAB A from a myosin of adult human atrium (a cardiac MHC). The conditions under which these MAhs were produced, purified and characterized have been described by Leger et al. [11], '1 heir specificity has been described in earlier works [16]. Their cross-reactions wiih myosin from foetal and adult cattle have been analysed [18].

The monoclonal antibody (NLC-Titin) ami connectin raised against human skeletal muscle conneelin was the clone FI49.9B9 from Novocastra. Desmin was revealed by a monoclonal antibody Dako clone D33, ref. M760. Antibodies anti proliferating cell nuclear antigen (pcna) and K.I67 came from Dako with respectively ihe reference pclO, M879 and M722. r. Bromo 2'deoxyuridine (Brdu) was revealed with the Bocringher kit (Sigma 144461 I). Before their utili/ation the different antibodies were tested on bovine muscle.

Immunocytochemistry

The proteins present at the different stages of foetal life were evidenced by immunofluorescence according to the procedure mentioned by Pons et al. [16]. The cells were fixed in 100% ethanul lor 5 min. and then were washed twice in PBS. I'remcubaiion to reduce non specific binding was performed for 20 min. in PBS+2%serum albumin bovin (BSA. Sigina) Then cells were incubaied with the first monoclonal antibody (L. R, F or A not diluted in PBS + 2% BSA) for 40 min at room temperature. After 2 washings with PBS. (he second antibody (rabbit anti mouse IgG labelled with dichlorotriazinylaminofluoresceine, Immunotech) diluted in PBS + 2% BSA was applicated for 40 min at room temperature. After washings with PBS, the cells were fixed with mowiol (Calbiochem 475904).

The number of cells positive for each antibody was count formyoblast(MB), for small myotubes with nuclei number inferior to 10(MTs), and for large myotubes with a number of nuclei superior to 10(MT1).

Phosphocreatine Kinase (CPK) activity

Cells were washed with buffered saline (PBS) pH 7.4 and stored at - 80°C. Cells were extracted in digitonin buffer, incubated for 5 min at room temperature and eentrifugated at 8000 g at 4°C for 10 min to eliminate the excess of digitonin. CPK activity was measured on the supernatant using the sigma kit C K 10.

Protein preparation

Cells were rinsed with PUS, scrapped in 2 ml PBS and centrifuged at 1000 g for 5 min at 4°C. The pellet was suspended in 5 ml of extraction buffer (0.5 M NAC1, 20 mM sodium pyrophosphaie. 50 inM Tris. 1 mM EDTA, ImM dithiothreiiol *(D* IT)). After a 15 min. incubation at 4°C. each sample was centrifuged for 5 min at 2500 g. Supernatant was then mixed with glycerol at a final concentration of 50% (v/v) and stored at - 20°C for several months. Protein concentration of the sample was determined according to Bradford [4], using bovine serum albumin at 1 mg.'ml (w/v) as standard.

Results

Numbers of nuclei

For both genetic types, the number of nuclei per unit surface counted after staining with Giemsa increased sharply from day 2 to 8 and then less markedly from day 8 (Fig la). It was slightly higher in DM than inN cells on days 2 and 4. similar in the two types on day 6 and lower in DM at later times.

Mononucleate cells

The number of mononucleate cells per unit surface in N increased strongly until D8 and then decreased (Fig Ih). Il was higher in DM cultures on days 2. 4 and 6- On day 8 it was higher in N; it was the same in DM and N on day 10 and then higher once more in DM, By day 12 the number of mononucleate ceils had fallen markedly in N, while it remained high in DM. Figure *2* illustrates the wide differences in the numbers of cells on days 2 and 4 between N and DM foetuses. It also shows that by day 2 the DM cells were already aligned, while the N cells had not yet reached that stage.

Cell cycle phases

The response to anti-pcna (Fig 3a) showed that on days 1 and 2 the number of proliferating cells was 2 to 3 time higher in the DM than in the normal foetuses. It fell in DM between day 2 and 4 and then became stable, while in N it increased up to day 6. The response to the anti-Brdu (Fig 3b) antibody showed that on days 1 and 2 the percentage of cells in phase S was higher in the DM. In the normal foetuses, this percentage increased between day 2 and 4 and then decreased The number of cells in phase M recog nized by the ami-Ki67 antibody (Fig 3c) was nil on day I in both N and DM cultures. It increased from day 1 to 6 in N foetus and remained stable mid lower in DM from day 2 to 6. All these results show that on days I and 2 of culture the DM cells proliferate more that N cells.

Percentage oj fusion

In N cultures the percentage of fusion increased biphasically. strongly between day 2 and 6, then levelling off between day 6 and 10 before increasing again strongly between day 10 and 12 (Fig 4a). In DM the increase ivrwccn day 2 and 12 was more regular. Except for days 8 and ! (I, v\ here DM and N had closely similar values, the percentage of fusion at all stages was higher in N. On day 2 ihis percenlage was still nil in DM, but was alreatlj 6% in N. Giemsa staining showed that there were fewer fusions in DM on days 6 and 8 (Fig 5). Also, the distribution, of **Il>c** nuclei inside the myotubcs was different in N and DM on days 8 and 10. On day S in N the nuclei were aligned at the centre of the myotubes. W'hile in DM the nuclei were distributed in groups. On day 10 the N nuclei were distributed a! the edges of the myotubes. similar to the pattern observed in muscle fibres. In DM this arrangement was mueh less marked (Fig 5). This difference illustrates a delay in the differentiation of DM cells compared to normal ones.

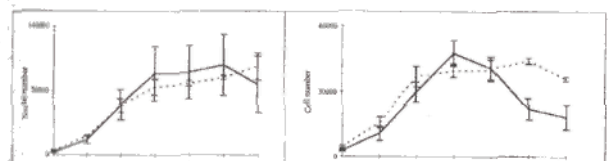

Figure 1. Evolution of the nuclei number (a) and monucleted t-elh (b) from 2 lo 14 days of cultures of normal — and-----double-muscled cells.

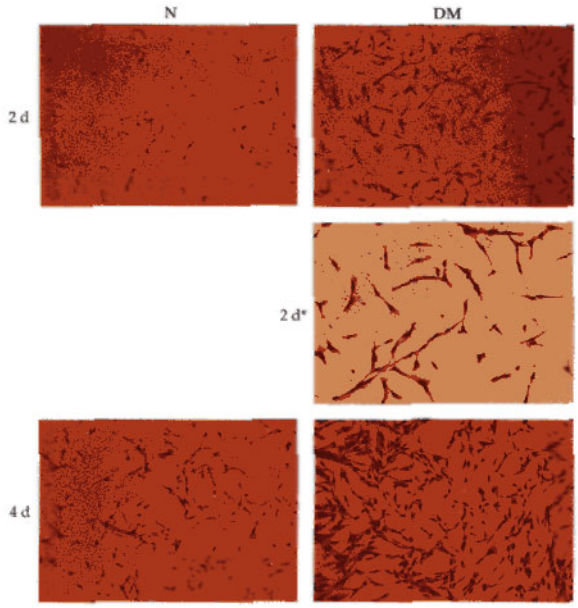

*Figure 2. Coloration of the nuclei of normal (N) andl double-muscled cells (DM) at 2 and 4 days in vitro. (x60; *:x 150).*

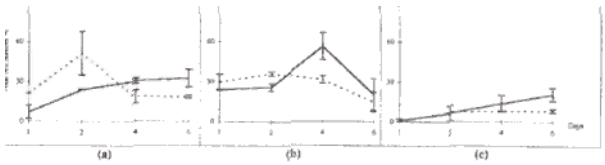

Figure 3. Study of the cell cycle vs normal — and double-muscled - - - cultures. Percentage of the nuclei recognised by amipcna antibody; (h)percentage oj the nuclei recognized by anli Brdn antibody: (c) percentage of the nuclei recognized by anti Ki67 antibody.

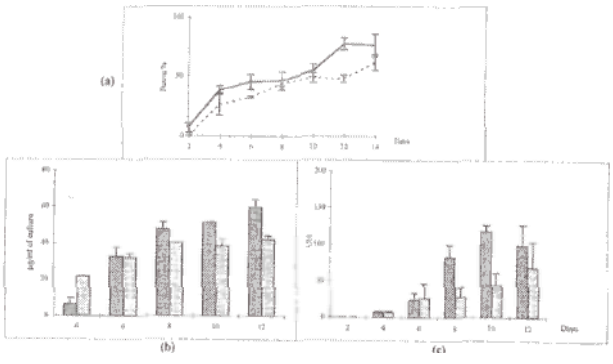

Figure 4. (u) percentage of fusion: (b) quantity of myofibrillar proteins; (c) Creatine phosphokinuse activity (CPK) of normal S and double-muscled i \ *cells at the different stages of primary culture.*

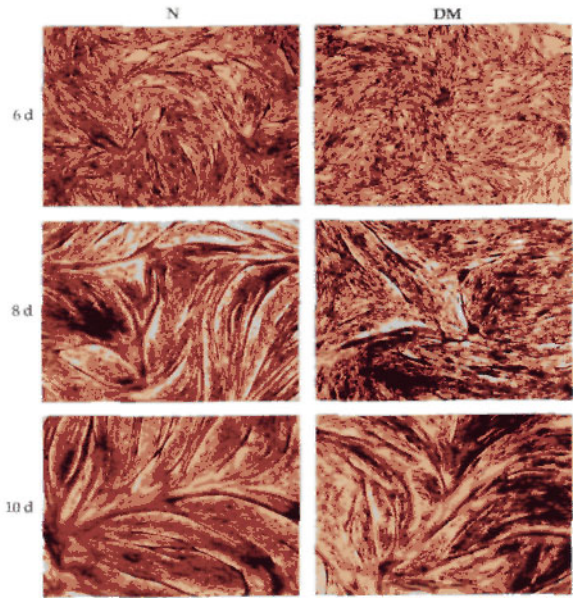

Figure 5. Nuclei coloration of normal (N) and double-muscled (DM) myotubes tit 6. 8 and !0 days of primary cultures <x60).

Myofibrillar proteins

The quantity of myofibrillar proteins (ug/ml) increased regularly between day 4 and 12 in N (Fig 4b), In DM it increased between day 4 and 8 and then became stable. On day 4 the quantity of myofibrillar proteins was much higher in DM (due to more cells in DM), on day 6 it was the same in DM and N, and subsequently it was higher in N.

Creatine phosphokinase activity

CPK activity (Fig 4c) was nil on day 2 in both DM and N, but then rose strongly in N, peaking on day 10, In DM this increase was much less marked. On days 4 and 6 the activity was the same in N and DM, and in contrast much higher in N than in DM at the other stages.

Im munacytochemisliy

Desmin

The number of myoblasts (MB) recognized by the anti-desmin antibody (Fig 6a) in N increased strongly between day 2 and 6, became stable between day 6 and 8, and then fell. In DM this value increased between day 2 and 8 and then fell. The number of desmin positive cells was thus twice as high in N on days 2, 4 and 6, and the same in N and DM thereafter. The number of desmin positive small myotubes (MTs) (Fig 6b) displayed a bellshaped lime course, peaking on day 8 in both genetic types. At all stages this number was higher in N, the difference being especially marked on day 8. The number of desmin positive large myotubes (MT1) (Fig 6c) in N rose strongly between days 4 and 6 and then fell moderately between days 8 and 10, and strongly between days 10 and 12. In DM the

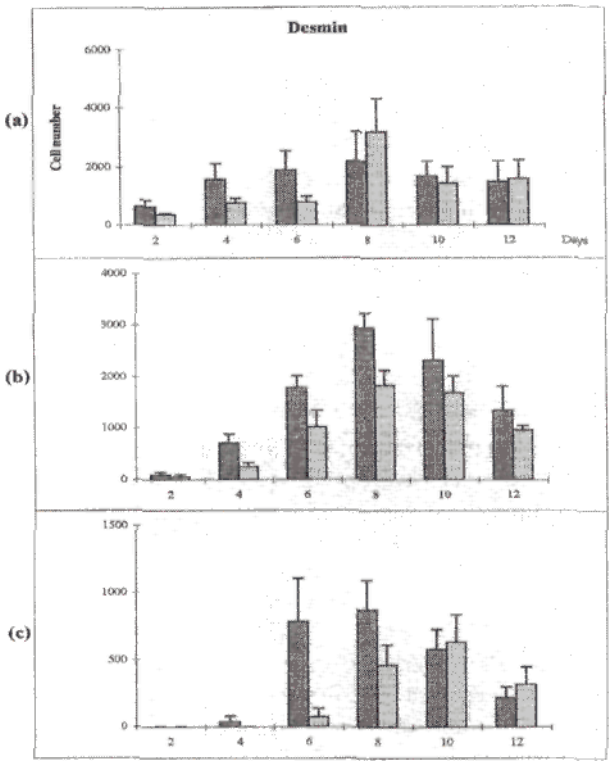

Figure 6. Proportion of desmin positive cells from 2 to 12 days of culture, iii (a) myobtasi: fh> small myotubes ami (c) large myotubes from normal tl and dotihle-mmcled O *cells,*

increase occurred only after day 6, peaking on day 10 and then falling.

Connectin

In the myoblasts, connectin appeared only on day 8, and then decreased, with no differences between the two types (Fig 7a). In small myotubes, connectin appeared by day 4 in N. increased strongly up to day 8 and then decreased (Fig 7b). In DM the rise was much less marked, peaking at day 10 and then falling. At all stages the proportion of connectin positive myotubes was greater in N than in DM, In the large myotubes (Fig 7c) connectin increased from day 6 in N. peaked at day 8 then fell. In DM it rose only from day 8 and peaked at days 10 to 12.

Slow MHO (MHC I)

in N the number of MB recognized by the anti-MHC 1 antibody increased from day 2 to 6 and then remained stable until day 12. In DM it rose only after day 4, peaking at day 8 and then falling. On days 2, 4 and 6 this number was lower in DM than in N, but thereafter there was little difference between N and DM cultures (Fig 8a). The number of MHC 1 positive MTs in N increased between days 4 and 10 and then diminished. In DM it rose especially from day 6, peaking on day 10 and then falling. Thus the number of MTs containing isoform MHC I was lower in DM at all the stages (Fig 8b). The number of MHCI positive MT1 increased from day 6 in N, peaking at day 8 and then falling. In DM it rose especially between days 8 and 10 and then fell. On day 8 the number of slow MT1 was

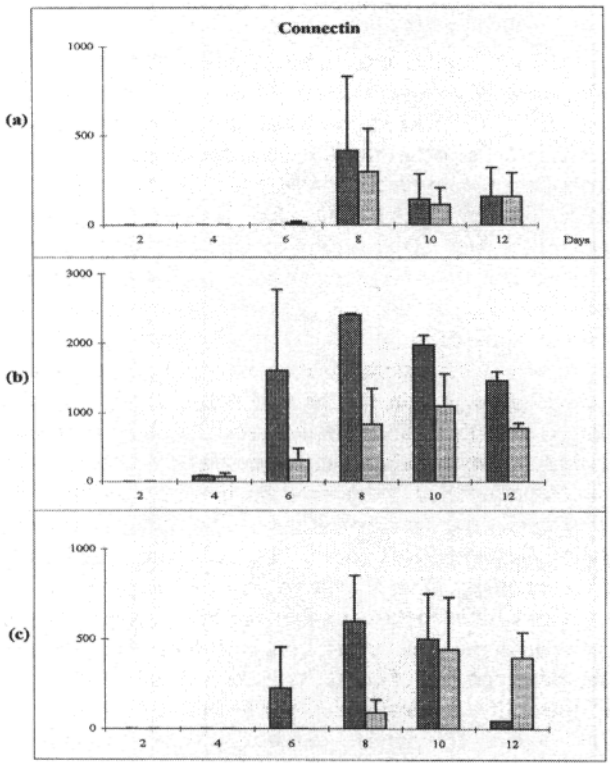

Figure 7. Proportion of connectin positive cells from 2 to 12 days of culture, in (a) myoblasts; (b) small myotubes and (c) large myotubes.

appreciably higher in N. Therafter the number was the same in N and DM (Fig 8c).

Foetal MHC (MHC F)

In N the number of myoblasts recognized by the anti-foetal myosin antibody increased strongly between day 2 and 6, diminished up to day8 and then became stable. In DM it was low on days 2 and 4, then increased and became stable beween day 6 and 12. At all the stages the number of MHCF positive MB was thus higher in N than in DM, especially on day 6 (Fig 8a). The number of MTs recognized by the foetal antibody increased in N between day 4 and 8, and then fell. In DM it increased especially between day 6 and 10 and then fell. Thus at all the stages the number of MHC F positive MTs was lower in the DM cells. (Fig 8b). The number of MT1 recognized by the anti-foetal antibody was nil in both genetic types on day 2 and 4. In N it was high on day 6, rose until day 8 and then fell, while in DM it rose strongly only between day 6 and 8, and then fell. Thus the number of MHC F positive MT1 was lower on days 6 and 8 in DM than in N (Fig 8c).

Fast MHC (MHC 2a and 2b)

In N, the number of myoblasts containing the fast MHC isoforms rose between day 4 and 6, peaking on day 10, and then fell (Fig 9a). In DM this isoform was absent at all the stages. In the MTs of N, the fast MHC rose between day 4 and 6 and became stable between 8 and 12 days. In DM it increased, though weakly, from day 6 (Fig 9b). In the MT1 of N, this isoform rose strongly between day 6 and 8 and then fell. In DM it was present in very small quantities on

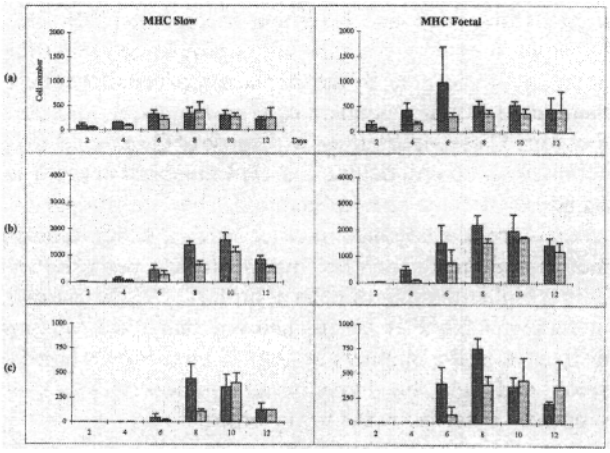

Figure 8. Proportion ofMHCslow or MHC foetal positive cells from 2 to 12 days of culture, in (a) myoblasts; (b) small myotubes and (c) large myotubes.

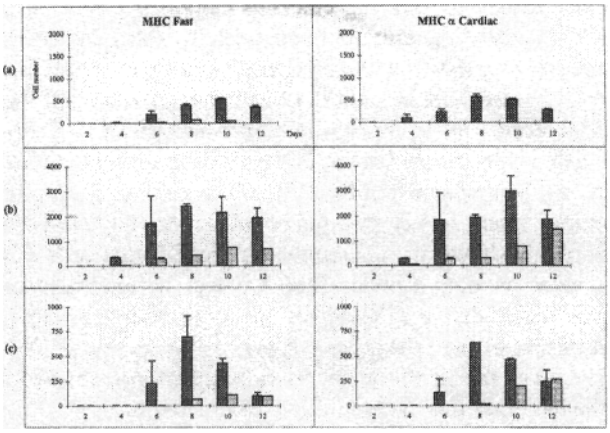

Figure 9. Proportion of MHC fast or MHC alpha cardiac positive cells from 2 to 12 days of culture, in (a) myoblasts; (b) small myotubes and (c) large myotubes.

days 8, 10 and 12 (Fig 9a). At all the stages and in all cell types, fast MHC was thus present in much larger amounts in N cells.

a-cardiac MHC (MHC A)

The a-cardiac isoform displayed a time course similar to that of fast MHC (Fig 9a). It was present only in the N myoblasts, peaking on day 8. In the MTs it increased from day 4 in N, peaking on day 10. In DM it rose weakly from day 6 to 12. At all the stages it was less abundant in DM (Fig 9b). Likewise, it rose on days 6 and 8 and then fell in the MT1 in N, while it increased only after day 8 in DM (Fig 9c). Like fast MHC, the a-cardiac MHC isoform was present in greater amounts in N foetuses at all stages and in all cell types.

Discussion

Cell proliferation phase

The muscles of adult double-muscled bovine are characterised by larger sizes due to both hyperplasia and hyper-

trophy (for review see King and Menissier, [10]). This hyperplasia evidently begins very early, since the results of this study show that by the second day of primary culture there are 1.7 times as many cells in the double-muscled foetuses. These results agree with those of Quinn et al. [17] obtained on 90-day double-muscled myoblast cultures in a study focused on cell proliferation. They are also consistent with results obtained in other species, which showed that animal breeds with fast muscle growth generally exhibit a higher number of cells at birth [8, 19]. In contrast, in turkey, Cherel et al. [6] showed that there was no difference in the numbers of cells at birth in two breeds, heavy and light, but hyperplasia was nevertheless subsequently observed in the heavy breed.

Auda-Boucher [2] showed *in vitro* that embryonic myoblasts from fast-growing chicken breeds multiplied more actively than those from slow-growing breeds. Likewise, Duclos et al. [7], showed that the satellite cells in these same fast-growing chickens exhibited more active DNA synthesis in culture. In contrast, in turkey, no difference was observed in the proliferative abilities of satellite cells in two breeds with slow and fast growths [2]. For double-muscled foetuses, the higher number of mononucleated cells on the second day may have several origins. It may be assumed that the DM cells enter into the proliferation cycle sooner than the N cells, since the anti-pcna antibody shows that the number of proliferating cells was greater on day 2 in the DM foetuses. It may also be concluded that the length of the proliferation cycle is different in the two genetic types, being shorter in DM. However, the results do not provide any information about the length of this cycle.

At the same time, the fact that the percentage of fusion was at all stages lower in DM than in N suggests several possible interpretations for the high number of mononucleate cells on day 2. These cells may be myoblasts with a lower fusion potential in DM than in N cells, or satellite cells, or fibroblasts. The weaker response to anti-desmin antibodies in the DM cells suggests that many of these cells are fibroblasts, since they are desmin-negative. However, the fact that these mononucleate cells display a clear alignment by the second day of culture argues in favour of bovine myoblasts with delayed desmin synthesis relative to normal myoblasts [7]. Such explanations have been advanced for other species such as the chicken, in which it has been shown that these mononucleate cells are not satellite cells but actually myoblasts. In bovine, Quinn et al. [17] have shown that the hyperplasia in the DM concerns only the myogenic cell line, and not the fibroblastic cell types.

Overall, these results show therefore that compared with N, the DM cells display a greater proliferating capacity, and prompter proliferating and alignment phases. This advance is apparently lost after alignment; the fusion of the DM myoblasts and muscle protein synthesis are delayed.

Cell differentiation phase

The delay in muscle differentiation in DM foetuses was confirmed by various criteria. In particular, desmin and connectin, which are considered to be early markers of muscle differentiation, were expressed later in both myoblasts and myotubes in DM. Likewise, the expression of the different myosin heavy chain isoforms was delayed in DM, adding to the results of Quinn et al. [17], who showed that the overall expression of myosin occurred later in DM cells than in N cells between 2 and 6 days of culture. This delay in the evolution of the myofibrillar proteins was consistent with the lower CPK activity and percentage of fusion for the DM cells. In addition, the results corroborate and complement those obtained *in vivo* in muscle samples taken at different stages of foetal growth in double-muscled and normal bovine. Picard et al. [15] who monitored the expression of the different myosin heavy chain isoforms (slow, fast, embryonic, foetal and alpha-cardiac), have shown *in vivo* that the muscles of double-muscled foetuses exhibit a delay in their contractile differentiation. This delay concerns both the first and second generation of cells. From approximately 200 days of foetal life, the first-generation is entirely slow in both DM and N. In contrast, the second generation, which appears later, displays a delay up to approximately 230 days. Apparently, the delayed differentiation of the DM is much less marked at the end of gestation [15]. Our *in vitro* results also show that at 10 and 12 days of culture, the differences between N and DM cells are less marked. This delayed differentiation is apparently not systematic in rapid-growth lineages in different species. In the chicken, Auda-Boucher [2] have shown that the embryonic myoblasts of a rapid-growth lineage, which multiply more actively than those of the slow-growing lineage, nevertheless differentiate identically.

These differences, i.e., higher cell proliferation and delayed differentiation in double-muscled muscles, may be the consequence of different mechanisms of regulation in the two genetic types. The results obtained suggest the implication of a growth factor that stimulates proliferation and inhibits differentiation. Quinn et al. [17] have shown that a medium in which DM foetus cells had been cultured displayed a higher myotrophic activity than a medium in which N foetal cells had grown. These authors suggested that the hyperplasia in DM may be controlled by the hyperproduction by fibroblasts of an unidentified myogenic factor of 42 to 43 kda, which can bind to both myoblasts and IGFI. They suggest that growth factors such as PDGF and FGF may stimulate the proliferation of myoblasts indirectly by inducing a paracrine production of this trophic factor.

The fact that DM cells were aligned early than N cells, and fused later could also be explained by different intrinsic properties of N and DM cells. We can suggest that extracellular matrix proteins necessary for myoblasts fusion appear later in DM cells than in N.

Conclusion

In conclusion, the overall findings reported here support the results of Quinn et al. [19] showing a phase of higher cell proliferation in double-muscled than in normal cells. They add the new finding that the proliferation phase and eell alignment occur earlier in double-muscled cells. This advance is subsequently lost, as the double-muscled cells exhibit a delay in muscle differentiation affecting all the proteins analysed. These differences could be the consequence of either regulatory factors different between the 2 genetic types or intrisic properties of cells.

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