Int. J. Dev. Biol. 34: 171-180 (1990)

Characterization of myogenesis from adult satellite cells cultured *in vitro*

ALBERT LE MOIGNE*, ISABELLE MARTELLY, GEORGIA BARLOVATZ-MEIMON, RAPHAËL FRANQUINET, AHMED AAMIRI, ERIC FRISDAL, YANN BASSAGLIA, GEORGES MORACZEWSKI and JEAN GAUTRON

Laboratoire d'Etude sur la Myogénèse et la Régénération Musculaire (MYREM), UFR de Sciences, Université Paris Val de Marne, Créteil, France

ABSTRACT We describe several characteristics of *in vitro* myogenesis from adult skeletal muscle satellite cells from the rat and several amphibian species. The timing of cell proliferation and fusion into myotubes was determined, and in urodeles, myogenesis from satellite cells was clearly demonstrated for the first time. Growth factors are known to stimulate satellite cell proliferation. Acidic FGF mRNA was present in rat satellite cells during proliferation but it was not detected in myotubes. Fibronectin was synthesized in satellite cells during proliferation and expelled into the extracellular medium when the myotubes differentiated. We suggest that fibronectin plays a part in the formation of myotubes, as this process was inhibited by anti-fibronectin IgG. Adult satellite cells might differ from fetal myoblasts since they were observed to exhibit the opposite response to a phorbol ester (TPA) to that of the myoblasts. We therefore examined the possibility that the different levels of protein kinase C activity and different phorbol ester binding characteristics in the two cell types account for these opposite responses. Our results suggest that the difference is not connected with the phorbol ester receptor but might be caused by events subsequent to protein kinase C activation. Localized extracellular proteolytic activity might have a role in cell mobilization and/or fusion when satellite cells are activated. We showed that the content of plasminogen activators, chiefly urokinase, was larger in tissues from slow twitch muscles which regenerate more rapidly than fast muscles. The urokinase level rose sharply in cultures when cells fused into myotubes, and was twice as high in slow muscle cells as in fast ones. We also found that, *in vitro*, slow muscle satellite cells displayed greater myogenicity, but that phorbol ester inhibited their mitosis and myogenicity. We conclude that satellite cells acquire characteristics which differentiate them from myoblasts and correspond to the fast and slow muscles from which they originate.

KEY WORDS: *cultures in vitro, satellite cells, aFGF, fibronectin, plasminogen activators, protein kinase C*

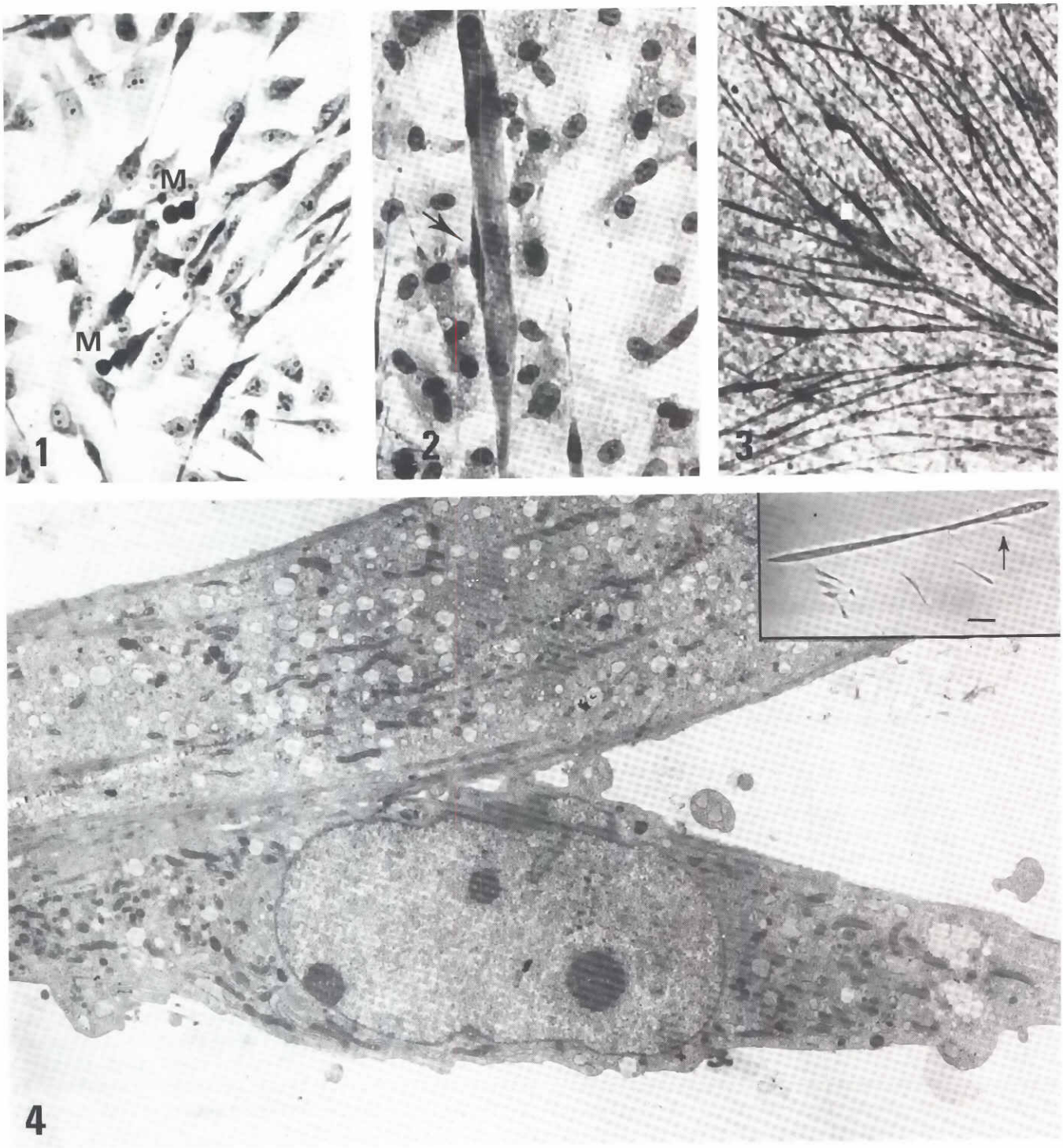
Introduction

In most tissues of adult organisms, cellular homeostasis is achieved by the continuous replacement of cells that have died or been lost after injury. In tissues like blood or squamous epithelia, which contain clearly identified stem cells, the cell turn-over is rapid and permanent, unlike skeletal muscles in which it is very slow. However, turnover may be activated by wounding, after which muscle tissues reveal great regenerating capacities (Carlson, 1973). A population of myogenic cells with the characteristic features of resting cells was first described in frog muscles (Mauro, 1961) and has since been reported in many vertebrates (review in Campion, 1983; Plaghi,1985). In intact muscles, these resting cells are wedged between the basement membrane and the plasmalemma of the myofibers and are therefore known as satellite cells. They can be activated during muscular growth and traumatic regeneration. In the latter case, they proliferate and either fuse to form new myotubes which differentiate into myofibers, or are

Abbreviations used in this paper: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; EDL, extensor digitorum longus; PDBu, phorboldibutyrate; PKC, protein kinase C; t-PA, tissue type plasminogen activator; TPA, phorbol ester 12-O-tetrade canoyl phorbol-13-acetate; UK, urokinase plasminogen activator

***Address for reprints**: MYREM, UFR de Sciences et Technologies, Université de Paris Val de Marne, Avenue du Général de Gaulle, F-94010 Creteil, France

0214-6282/90



Figs. 1-4. Histology of *in vitro* myogenesis. (1) *4-day rat myogenic cell culture before the beginning of cell fusion. Giemsa staining (x450). M: Mitosis.* **(2)** *6-day culture; early myotube with a cell in "satellite-like" position (arrow). Giemsa staining (x450).* **(3)** *Low magnification view of a 14-day culture showing a network of myotubes. Giemsa staining (x150).* **(4)** *Electron micrograph of fusion between the early myotube and the satellite cell indicated by an arrow in the inset. Distinct remnants of the myotube and the satellite cell membranes are still visible in the contact and consecutive fusion area (x4400; inset x100).*

incorporated into preexisting fibers (Bischoff and Holtzer, 1969; Moss and Leblond, 1971; Lipton and Schultz, 1979; Bischoff, 1986).

These myogenic processes, which start in adults with the activation of satellite cells and end with myofiber differentiation, are not completely understood. The fact that these cells are scattered

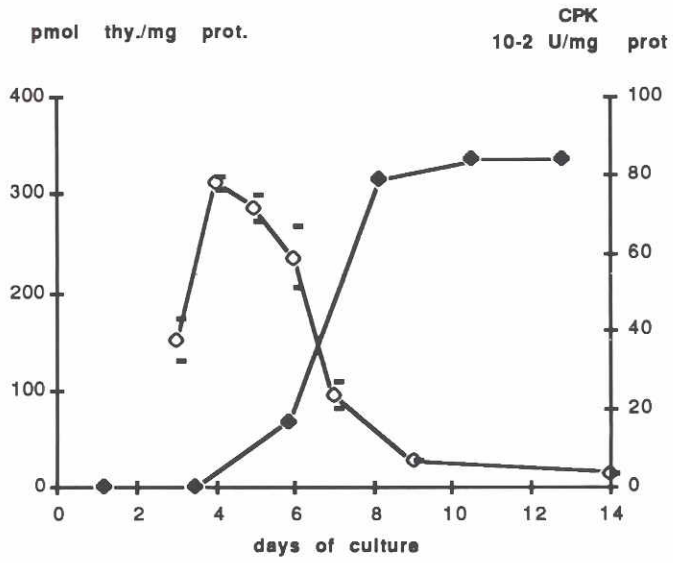


Fig. 5. Variations in DNA synthesis and in muscular creatine phosphokinase in rat satellite cell cultures. *DNA synthesis was measured after 6h incubation with ^{3}H-Thymidine. M-Creatine phosphokinase was measured by the level of creatine phosphate present in culture.*

among the muscle tissues complicates certain approaches. Isolation of satellite cells from muscle tissues and their culture *in vitro* is a procedure that facilitates the study of the first steps in myogenesis and has therefore been adopted by several laboratories (see for instance Bischoff, 1974; Askanas and Engel, 1975; Konigsberg, *et al.*,1975; Allen *et al.*, 1980).

Although these cells have been widely identified and studied in mammals, birds and anuran amphibians, there is still controversy concerning their role in the muscles of urodele amphibians which regenerate a complete limb or a tail after amputation. In this respect, histological or immunocytochemical studies of regenerates have not allowed a distinction to be made between degenerative and regenerative processes, particularly as regards the identification of the cells responsible for muscle regeneration (Hay, 1968; Lentz, 1969; Kintner and Brockes, 1984).

We are at present studying the different steps in myogenesis in primary cultures of satellite cells in parallel to *in vivo* investigations in progress in our laboratory. For this purpose we are culturing adult rat satellite cells with a technique described by Hantai *et al.* (1985) and Gautron (1988), which has been adjusted to the muscle cells of amphibians, *Xenopus* (Franquinet *et al.*, 1988) and two urodeles (Franquinet, 1988).

The present review starts with a brief chronological description of *in vitro* myogenesis followed by a definition of the proliferation and the fusion phases which will be further analysed in rat and amphibians cells. As regards the latter, successful cultures of urodele satellite cells should help to clarify the long standing controversy on limb muscle regeneration. We then go on to summarize the results of several studies of the factors involved in cell proliferation and myofiber differentiation. Satellite cells synthesize growth factors and fibronectin, a component of the extracellular matrix which seems to play a role in cell fusion. Next, we compare satellite cells to myoblasts, from which they might differ in their sensitivity to tumor promoters. Our review ends with a commentary on several results which indicate that satellite cells might receive imprints corresponding to the slow or fast characteristics of the myofibers that they accompany.

Cell proliferation and myotube differentiation *in vitro*

Rat satellite cell cultures

Muscle dissociation and isolation processes were found to release a cell population consisting mostly of satellite cells, which were grown in primary cultures, as described in Gautron (1988).

In these cultures, rat myogenic cells adhered to the substrate within a few hours and their number doubled every day until day 4 (Fig. 1); after that, their growth slowed down as the satellite cells began to align and fuse into myotubes (Figs. 2-3), so that from days 4 to 10, the cell population only doubled once. By day 14, a network of contractile myotubes had differentiated (Fig. 3). DNA synthesis was routinely measured using ^{3}H- thymidine incorporation by the cells. Labeling increased rapidly from the first day of culture, culminated at day 4 and gradually diminished to a low level at day 7 (Alterio, *et al.*, 1990). Myotube differentiation was evaluated by the usual method of measuring muscular creatin phosphokinase, a muscle specific enzyme (Lough and Bischoff, 1979). It appeared from day 4 of culture and reached a plateau at day 8 until the end of culture at day 14 (Fig. 5).

Cell proliferation was also carefully studied by image analysis of Feulgen-stained cultures using a SAMBA 200 microscope processor (Thomson TITN) as described in Brugal and Chassery (1971). The nuclei were automatically analyzed by calculating 18 parameters relating to the texture and densitometry of the chromatin, and the shape of each nucleus. Classes corresponding to the G0, G1, S, M and G2 cell cycle phases were determined. Cells from 0 to 14 day cultures were analyzed and classified according to the phase they had reached in the cell cycle (Fig. 6) (Lassalle *et al.*,1989; Le Moigne *et al.*, 1989). The proportion of cells in the S and G2 phases reached 42% by day 3 and gradually diminished from day 4, when the myotubes differentiated.

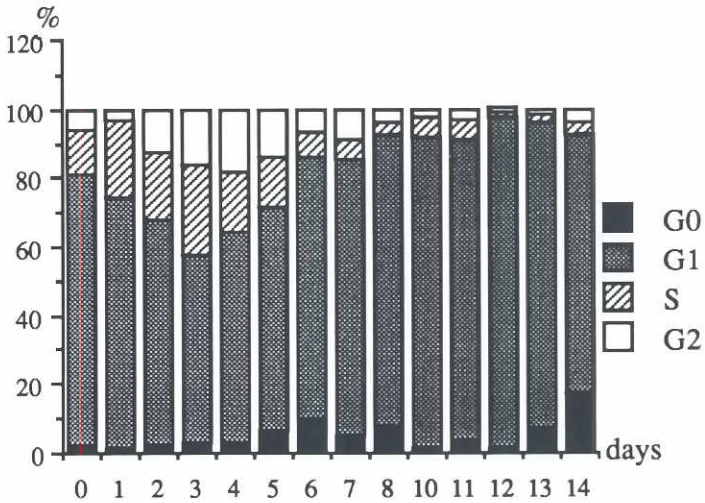


Fig. 6. Variations in the percentage of cells *in G0, G1, S, and G2 phases during 14 days of rat satellite cell cultures obtained from image analysis.*

Image analysis of early rat growing myotubes showed that the nuclei gradually passed from G1 into G0 from the extremities to the middle part of a myotube and acquired characteristics resembling those of adult myofiber nuclei (Lassalle *et al.*, 1989). Ultrastructural micrographs of the fusion between a myotube and satellite cells showed that before fusion the membranes gradually disappeared in the contact area (Fig. 4). A cinematographic study of rat satellite cell cultures (Pouchelet and Barlovatz-Meimon, 1989), showed that satellite cells entered into contact with the myotubes several times before the final fusion, as if the latter required preliminary events at the membrane level. The last contact, which ended in fusion, induced important cytoplasmic movements in the region of the myotube into which the cell was destined to be incorporated.

Under our conditions, we estimated that the proportion of non myogenic cells was, at most, 12% to 14% of the total number plated. This proportion was counted in experiments in which cells obtained from dissociated muscles were plated in 96 multiwell dishes at the concentration of 1 cell per well (Lassalle *et al.*, 1989). At day 11 after plating, each clonal culture was stained and examined for myotube detection. We considered that wells in which mononucleated cells proliferated but did not differentiate into myotubes had been initially plated with non-myogenic cells.

The biochemical measurements already described corroborated the results of image analysis; accordingly, we focused our subsequent experiments on rat myogenesis on days 3-4 for the proliferative phase and on days 12-14 for the differentiation phase.

Amphibian satellite cell cultures.

In amphibians, *in vitro* proliferation and differentiation were slower than in the rat as *Xenopus* satellite cells only adhered to the dish substrate at day 3 instead of a few hours after plating, and proliferation reached its peak at day 8 versus day 4 in the rat cells. By day 8, 75% of the nuclei were labeled after 24h incubation with ^{3}H thymidine, a proportion which then decreased until day 30. The first myotubes were seen at day 10, by which time they had only incorporated 5% of the nuclei (Franquinet *et al.*,1988). The slowest dynamics of myogenesis were those observed in the urodele species, in which the isolated satellite cells did not adhere to the substrate until day 6. After that, their mitotic index rose until day 15. The first myotubes were seen after day 20 in *Amblystoma* cultures and after day 25 in *Pleurodeles* cultures. These primary cultures also included about 12% of non-myogenic cells (Franquinet 1988).

The fact that we succeeded in isolating and culturing urodele myogenic mononucleated cells is interesting in the context of limb regeneration. It was generally accepted that when urodele limbs regenerated, the cells of the blastema resulted from the dedifferentiation of stump tissues (for review, see Hay, 1968; Kintner and Brockes, 1984). The multinucleated myotubes of the stump muscle were then believed to break up into mononucleated cells which were reported to proliferate and fuse again to form myotubes (Lentz, 1969). The existence of satellite cells in post-metamorphic urodele muscles was contested by several authors (Hay and Doyle, 1979); however, Popiela (1976) provided good evidence for the presence of satellite cells in urodeles. When newt muscle tissues were dissociated and cultured *in vitro* without previous separation of fibers and mononucleated cells (Schrag and Cameron, 1983), cell fusion and myotube differentiation were obtained. However, this technique raised some doubts about the origin of myogenic cells.

We obtained a definite answer from the results of our satellite cell cultures of larval and adult *Pleurodeles* and *Amblystoma* in which the cells were carefully isolated from the myofibers (Franquinet, 1988,1989). As reported above, *in vitro* myogenesis progressed more slowly in urodele than in *Xenopus* cells. Curiously, larval *Axolotl* cells did not fuse into myotubes under our experimental conditions.

Further ^{3}H-thymidine labeling experiments and cell counting revealed that the number of cells doubled every three days after adhesion to the substrate. The fusion rate rose from day 7 in *Amblystoma* and days 12-15 in *Pleurodeles*, giving elongated myotubes which measured up to 1mm after 30 days of culture. Proliferation and fusion remained considerable after several replatings, and we calculated that after 2 months of culture and 4 replatings under clonal conditions, the entire lineage of a single cell was theoretically 2^{32} cells.

We cultured the myofibers of the urodele muscles we had isolated for cell extraction in order to verify that they did not fragment into mononucleated cells. Our cultures remained alive for 30 days, without any fragmentation or nuclear division. These results, which were expected, were the opposite to those obtained in the earlier *in vivo* observations mentioned above.

We concluded that urodele muscles possess satellite cells with sufficient myogenicity to allow limb muscle regeneration.

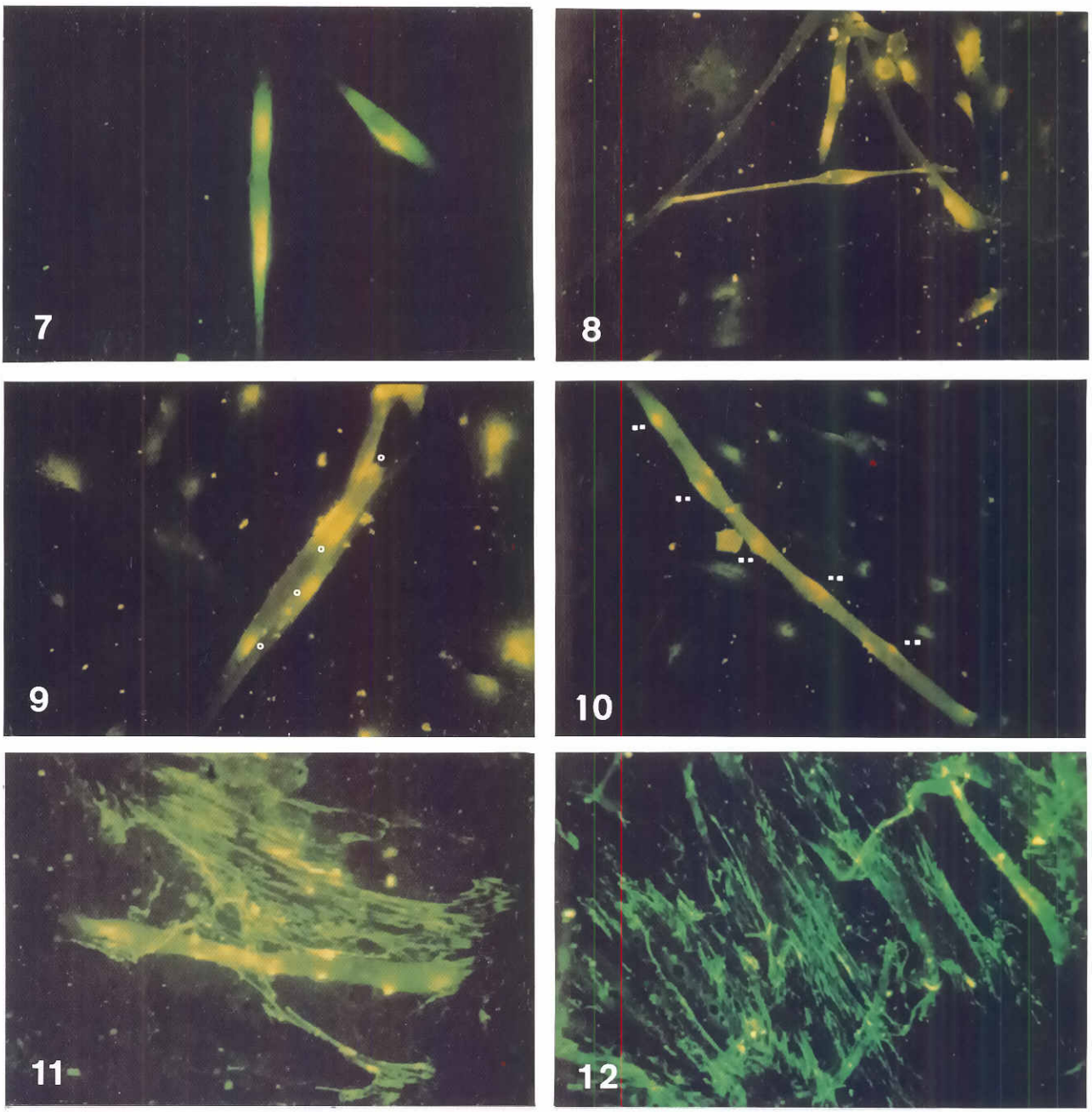
Factors involved in satellite cell proliferation and myotube differentiation

Expression of acidic FGF mRNA in satellite cells

The proliferation of mesodermal tissue is known to be regulated by FGF (Lathrop *et al.*, 1985a and b) especially basic FGF (bFGF) (Munaim *et al.*, 1988). The proliferation of satellite cells *in vitro* is stimulated by both basic and acidic FGF(aFGF) (Allen *et al.*, 1985; Bischoff, 1986). This stimulation was confirmed in our observations (unpublished). In addition, bFGF was detected in *mdx* mice mutants, whose muscular tissues regenerate continuously, at much higher concentrations in the basement membrane of muscle fibers than in non-dystrophic muscles (Di Mario *et al.*, 1989). As the origin of this growth factor is widely misunderstood, we attempted to establish whether satellite cells synthesize bFGF, at least partially.

The mRNA we extracted for this purpose from cultured cells was purified and hybridized with ^{32}P-labeled cDNA probes from bovine aFGF mRNA (Alterio *et al.*, 1988) and bovine bFGF mRNA (Abraham *et al.*, 1986). We extracted the mRNA studied on day 4 from cultures containing proliferating cells and on day 14 from those containing myotubes. Hybridization was detected between mRNA from the 4-day cultures and aFGF probe, but none was observed with mRNA from the 14-day cultures. Under the same conditions, weak hybridization was detected between bFGF cDNA probe and mRNA from 4day cultures (Alterio *et al.*, 1990). The presence of aFGF mRNA coincided with that of aFGF protein that was at its maximum of concentration in proliferative cultures whereas this factor almost disappeared from differentiated myotubes (Groux-Muscatelli, personal communication). This arrest of aFGF mRNA synthesis at myotube differentiation might correspond to the inhibitory effect of exogenous FGF on myoblast differentiation reported by several authors (review in Florini and Magri, 1989).

To sum up, proliferating satellite cells synthezise, at least in part, FGF, especially aFGF; this synthesis ceases at differentiation.



Figs. 7 to 12. Immunofluorescent labeling of fibronectin in *Xenopus* satellite cells cultures. *Fixed cultures were incubated in sheep IgG directed against human fibronectin, and fibronectin was revealed with fluorescent rabbit anti-sheep IgG (x450).* **(7)** *Fibronectin located at the membrane surface of isolated cells in an early culture, before cell fusion.* **(8)** *Migrating satellite cell showing intense labeling at the extremities in contact with early motubes.* **(9)** *Fibronectin distributed in contact areas (o) between the membranes of several satellite cells aligned before fusion.* **(10)** *Distibution of fluorescent patches of fibronectin on the membrane surface of an early myotube (▪▪).* **(11)** *Immunofluorescent labeling of an early myotube in which positive spots are scattered along membranes. On the left, the extracellular matrix is labeled.* **(12)** *Extracellular network of fibronectin in a 16-day culture, in which myotubes are no longer fluorescent.*

These results suggest that aFGF as well as bFGF might play a role in myogenesis, possibly through autocrine activity.

Extracellular matrix organization and satellite cell fusion

Satellite cells in culture form alignments and then fuse into myotubes. The extracellular matrix might be involved in these processes. Fibronectin is the main constituent of cell migratory pathways in morphogenesis (Thiery *et al.*, 1982; Boucaut and Darribère, 1983) and the migratory movements of cultured cells (Rosario *et al.*, 1983). Its presence is necessary for *in vitro*

TABLE 1

VARIATIONS IN THE LEVEL OF FIBRONECTIN SYNTHESIZED FOR 24 h IN 6 DAY MONONUCLEATED CELL CULTURES AND 16-DAY MYOTUBE CULTURES

	dpm/mg protein	dpm/mg DNA
6 days	3490	2970
16 days	2388	2897
Variation	-32%	-3%

Note: Fibronectin was labeled with ^{35}S-methionin for 24h and immunoprecipitated with anti fibronectin IgG. Results are expressed as dpm/mg DNA and as dpm/ mg protein.

myogenesis (Hauschka, 1966; Chiquet *et al.*, 1979, 1981; Ehristman *et al.*, 1981) and it is close to the myotubes on lateral adhesion sites (Gardner and Farmbrough, 1983). However, the question of whether, in satellite cell cultures, the origin of fibronectin is myogenic is still controversial. We studied the localization of fibronectin in adult *Xenopus* satellite cell cultures using immunofluorescence techniques (Aamiri *et al.*, submitted), which indicated specific fibronectin binding to cell membranes in isolated cells (Fig. 7). This fluorescence became stronger and occurred in adjacent membranes when the cells formed alignments just before fusion (Fig. 8-9). In early myotubes, fluorescent labeling of the membranes was distributed as spots (Fig. 10-11); on the walls of late myotubes, it decreased and finally disappeared. At that stage, fibronectin labeling was exclusively located in an extracellular framework in which the large myotubes were embedded (Figs. 11-12). Furthermore, when membranes were permeabilized before labeling, satellite cells and early myotubes revealed a cytoplasmic fluorescence which disappeared in late myotubes. The low fluorescence of non-myogenic cells remained unchanged throughout the experiment time.

The myogenic origin of at least part of the fibronectin was confirmed when its synthesis was measured after ^{35}S-methionine labeling and immunoprecipitation with antifibronectin IgG in cultures of satellite cells from rat (Hantai *et al.*, 1985) and *Xenopus* (Aamiri *et al.*, submitted). In the *Xenopus* cells, the amount of fibronectin synthesized was estimated per units of DNA and of protein in the cultures (Table 1). When assessed as the amount synthesized per nucleus, it remained constant between day 6, during the proliferation phase, and day 14, after differentiation into myotubes. However, when considered as part of total protein synthesis, fibronectin synthesis diminished when cytoplasm became abundant in the myotubes. The distribution of ^{35}S labeled fibronectin between the cytoplasmic, membranous and extracellular fractions was studied by an immunoprecipitation technique. At day 6, before cell fusion, fibronectin was principally cytoplasmic and membranous, but at day 14, after myotube growth, most of it became extracellular (Table 2). These changes in the distribution of synthesized fibronectin among the three fractions were consistent with the changes described above in the pattern of immunofluorescent-labeled fibronectin. A similar pattern of fibronectin synthesis was observed in rat cell cultures, and parallel variations were measured in the ratios of fibronectin synthesis in undifferentiated satellite cells and in myotubes, as well as changes in the distribution of this molecule between the cellular and matrix compartments (Hantai *et al.*,1985).

TABLE 2

COMPARISON BETWEEN THE DISTRIBUTION OF THE FIBRONECTIN SYNTHESIZED IN 6-DAY MONONUCLEATED CELL CULTURES AND IN 16-DAY MYOTUBE CULTURES

	6 days	16 days
DOC extract	42%	6.5%
Urea extract	26%	14.5%
Culture medium	32%	79%

Note: The synthesized fibronectin, labeled as mentioned in Table 1, was distributed between the culture medium, deoxycholate (DOC) extracts, which constitute the cytoplasmic fraction, and Urea extracts which represent the membrane component.

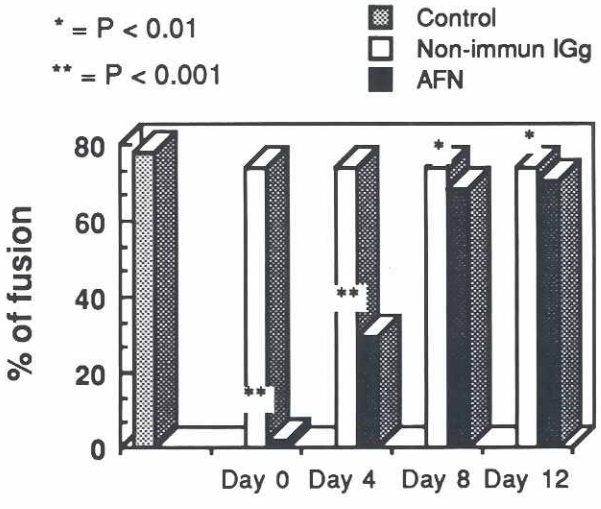


Fig. 13. Chart showing the cell fusion rate at day 16 *in* Xenopus *satellite cell cultures to which antifibronectin IgG (AFN) or non-immune IgG were added at different times.*

Changes in fibronectin distribution seemed to be related to cell fusion. The first data supporting this idea were obtained from satellite cell cultures in the presence of IgG directed against human fibronectin: in these cultures, cell fusion was inhibited, and the earlier the IgG was added to cultures, the greater the inhibition of fusion (Fig. 13). The same inhibition of cell fusion was observed when RGD peptide, which binds competitively to fibronectin receptors, was supplied to the cultures. However, when culture dishes were coated with exogenous fibronectin, cell fusion was accelerated (Aamiri *et al.*, in preparation).

We concluded from these experiments that most of the fibronectin present in our cultures was synthesized in myogenic cells and early myotubes. The changes observed in fibronectin distribution might be related to modifications in the distribution and number of the membranous fibronectin receptors such as integrin which are bound to cytoskeletal proteins. Although the changes in the relationships between fibronectin and the cytoskeleton during satellite

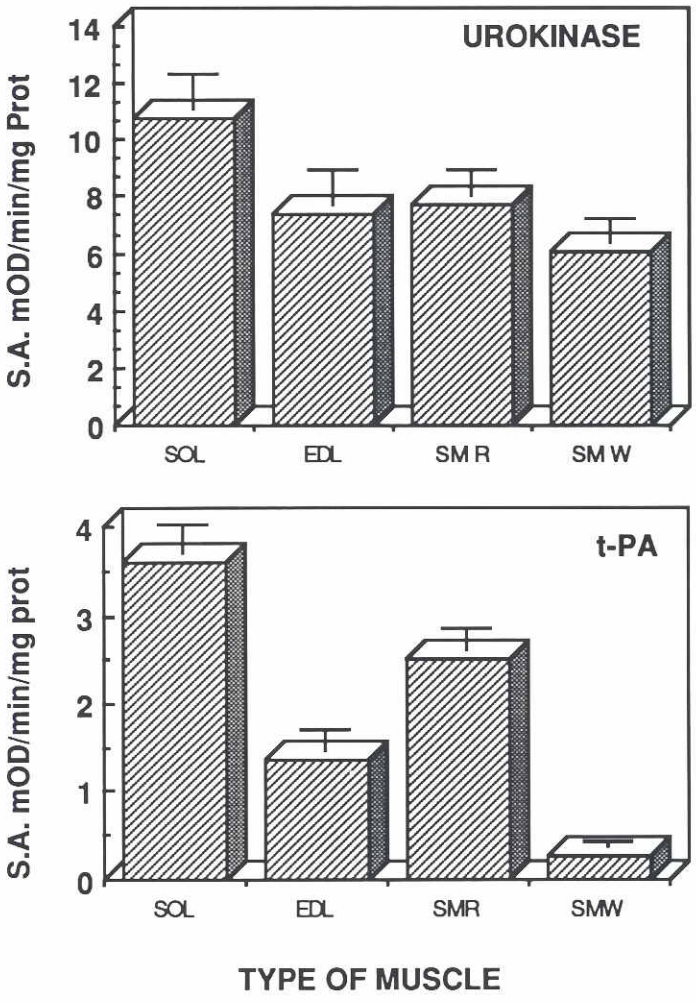


Fig. 14. Urokinase and t-PA contents of skeletal muscle extracts. *Extracts were obtained as described in Barlovatz* et al. (1990). *To measure urokinase content, the assay was performed in the presence of 400 nM plasminogen and 1.6 mM of the synthetic substrate S-2251. Plasmin generation was detected by measuring p-nitroaniline release from the substrate. To measure t-PA content, fibrin-bound t-PA was determined in the presence of 0.2μM of plasminogen and 0.6mM of the synthetic substrate CBS 3308. Values are expressed in specific activities (mOD/min/mg prot). Both slow Soleus and fast EDL muscles contained more UK than t-PA but the amount was larger in* Soleus, *indicating greater myogenicity.*

cell fusion are not yet understood, we conclude that fibronectin plays a crucial role in the early phases of alignment and fusion.

Plasminogen activators and cell fusion

After injury and/or denervation, muscle fibers degenerate and atrophy *in vivo* whereas satellite cells are activated and proliferate. Concomitantly with cell proliferation, great changes in the basement membrane are observed (Gulati *et al.*, 1983). Consequently, an important question affecting *in vivo* and *in vitro* myogenesis is the possibility that proteinases control these changes, which is why we studied the activity of plasminogen activators (Barlovatz-Meimon *et al.*, 1990).

This idea of enzymatic control has been put forward by several authors and was strengthened by Festoff *et al.* (1986) who demonstrated that plasmin is capable of degrading most of the basement membrane components after muscle denervation. These changes depending on the plasminogen-plasmin system are in agreement with this system's involvement in other tissue remodeling (Danø *et al.*, 1985).

We verified that similar stimulation of plasminogen activators occurred after wounding, as satellite cell mobilization is necessary for muscle regeneration. For this purpose, we compared the activities of plasminogen activators in two antagonist contracting hind limb muscles, slow twitch *Soleus* and fast twitch *Extensor Digitorum Longus* (EDL), and also in a composite muscle, the sternomastoid which has a slow twitch white part and a fast twitch red part. This was done because fast and slow muscles differ in several respects such as innervation and regeneration, which is faster in slow muscles. Consequently, the activity of plasminogen activators might differ in each type of muscle. To verify this, we assayed the activities of urokinase (UK) — and tissue type (t-PA) plasminogen activators in muscle extracts, by fibrin zymography and liquid and solid phase fibrin spectrophotometry. The muscle extracts expressed amidolytic activity and contained UK (38 Kd) and t-PA (70 Kd). Slow muscles contained larger amounts of both activators than fast muscles as *Soleus*/EDL ratio was 1.86 for UK and 1.82 for t-PA, and the relative amount of UK was larger than that of t-PA in both muscles (Fig. 14).

We then attempted to identify differences between myogenic satellite cells which might be due to the type of muscle (slow or fast) from which they were isolated. In both *Soleus* and EDL cultures, we measured UK and t-PA activities during cell proliferation and after fusion into myotubes. They did not change significantly during proliferation but rose sharply from day 8, when fusion had commenced (Fig.15). We concluded that plasminogen activators might be involved in membrane remodeling at the time of fusion. In both series of cultures, the activity of UK was higher than that of t-PA, but these activities exhibited parallel rises during myogenesis. The ratio of slow *Soleus* to fast EDL activities for both plasminogen activators in culture was constantly about 2/1 (not shown).These results are consistent with the above report that *in vivo,* UK and t-PA activities were higher in slow muscles, which regenerate faster (Fig. 14). Furthermore, they support the idea developed below that satellite cells "remember" the type of muscle from which they originated.

Satellite cells and embryonic myoblasts

In the early embryo, somitic cells from myotomes, which are fetal myoblasts, migrate and proliferate in muscular buds. Some of these cells fuse into primary myotubes, and the remainder align along these myotubes and become surrounded by a basement membrane. Most of these myoblasts later fuse into secondary myotubes which in 17-18 day pregnant rats were found to differentiate into myofibers. Those that do not fuse become the satellite cells. During the same period, innervation develops and typing of myofibers takes place. According to their ratio of slow to fast fibers, muscles are classified as slow, fast or mixed fast-slow.

Protein kinase C activity and phorbol ester binding

The way in which fetal myoblasts are transformed into satellite cells at the end of embryonic development is still unclear. One basic

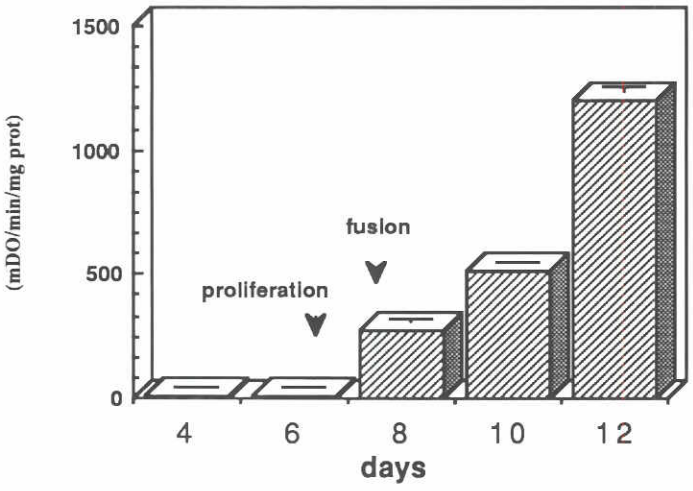


Fig.15. Intracellular urokinase content as a function of *in vitro* myogenesis. *UK content was determined as explained in Fig.14. UK was present in cultures at a low level during the proliferating stage and then rose strikingly at day 8 when mitoses had diminished and cells had begun to fuse.*

question is why satellite cells, which display myogenic potentialities after activation, normally remain in a satellite position in the muscle, instead of being incorporated into myofibers during development, in the same way as fetal myoblasts. As regards their myogenic capacities, satellite cells and fetal myoblasts seem to be very similar, as they can fuse with each other and form hybrid myotubes. However, an interesting difference has been described between these two myogenic cell types (Cossu *et al.*, 1983, 1985): when fetal myoblasts were treated with the phorbol ester 12-0-tetradecanoylphorbol 13-acetate (TPA), they did not differentiate *in vitro*, nor did they fuse into myotubes, but remained in a permanent proliferating state. In contrast, satellite cells from adult muscles were not affected by TPA treatment, which neither prevented the fusion process nor enhanced the proliferative capacities of these cells. The authors concluded that these differences argued in favour of the idea that satellite cells constitute a subpopulation of myogenic cells which emerge from the myoblasts during embryonic development.

Phorbol esters exert their effects by binding to their specific receptors located primarily in the plasma membrane. These receptors have been indentified as molecules of protein-kinase C (PKC) (Castagna *et al.*, 1982; Niedel *et al.*,1983). As their activation may account for most of the tumor-promoting effects of phorbol esters, it was suggested that the differential levels of PKC activity and/or phorbol dibutyrate (PDBu) binding to myogenic cells explained the differences observed between the sensitivity to phorbol esters of fetal and adult myogenic cells.

Accordingly, we compared the cellular distributions of PKC and the variations in PDBu binding in primary cultures of fetal myoblasts, adult muscle satellite cells and the L6 rat myogenic cell line (Martelly *et al.*, 1989). This line was a useful standard for interpreting our results as it is free of non-myogenic cells, unlike primary cultures of myoblasts and satellite cells, where non-myogenic cells are still present. These three types of myogenic cells were studied in the proliferative state and after fusion into myotubes. In all three, PKC activity was detected in both the membrane and cytosolic fractions prepared from primary cultures. The levels of PKC-specific activity were comparable in adult and fetal cells in the proliferating and differentiated states (Table 3). Total PKC activity diminished with fusion, and some of this activity was translocated from the membrane to the cytosolic compartment.

Binding of ^{3}H-PDBu to intact myogenic cells was also assayed. This binding increased with rising concentrations of the ligand and appeared to be saturable. This ^{3}H-PDBu was displaced by competition with TPA, but another phorbol ester (4-ß phorbol-12 13-didecanoate, 4-α-phorbol-12 13-didecanoate) which is not a tumor promoter, failed to bind to these cells.The Kd value of PDBu binding, which was between 6 to 13 nM in proliferating cells, rose to between 30 and 52 nM in myotubes (Table 4). Simultaneously, a small increase was observed in the total number of PDBu binding sites, as binding sites saturation (B max in table 4) increased by a factor 2 to 4 in the myogenic cells that we tested. These results prove that PKC activity and PDBu binding are similar in adult satellite cells and

TABLE 3

DISTRIBUTION OF PROTEIN KINASE C IN CULTURED MYOGENIC CELLS

	Protein kinase C		
	particulate	soluble	ratio
Satellite cells			
proliferating	56.4 ± 7.2	61.9 ± 9.0	0.91
myotubes	22.2 ± 4.3	54.0 ± 8.5	0.40
Fetal cells			
proliferating	52.2 ± 6.1	78.8 ± 10.3	0.76
myotubes	18.3 ± 4.9	61.5 + 9.4	0.30
L6 cells			
proliferating	19.2 ± 0.5	17.0 ± 0.9	1.13
myotubes	8.0 ± 0.2	20.4 ± 0.7	0.39

Note: Protein kinase C activity expressed in picomoles ^{32}P x min^{-1}x mg protein^{-1} was determined on particulate and soluble fractions of satellite cells from 2 month-old rats, on fetal myogenic cells from 17-day foetuses and on the L6 myogenic cell line. Values are means ±SE of seven and four separate determinations, made in duplicate on the satellite cells and the other two cell types respectively.

TABLE 4

CHARACTERISTICS OF ^{3}H-PDBU BINDING TO INTACT MYOGENIC CELLS

	Proliferating cells	Myotubes
Satellite cells		
Kd	10.0 nM ± 3.	42.1 nM ± 4.5
Bmax	0.95 ± 0.01	2.1 ± 0.20
Fetal cells		
Kd	4.8 nM ± 0.5	37 .3 nM ± 5.7
Bmax	0.23 ±0.05	1.01 ± 0.14
L6 cells		
Kd	8.0 nM ± 2.5	52.8 nM ± 9.8
Bmax	0.12 ± 0.03	0.38 ± 0.05

Note: B_{max} is expressed in pmol x mg protein^{-1}. Values are means of three to six separate determinations performed in duplicate.

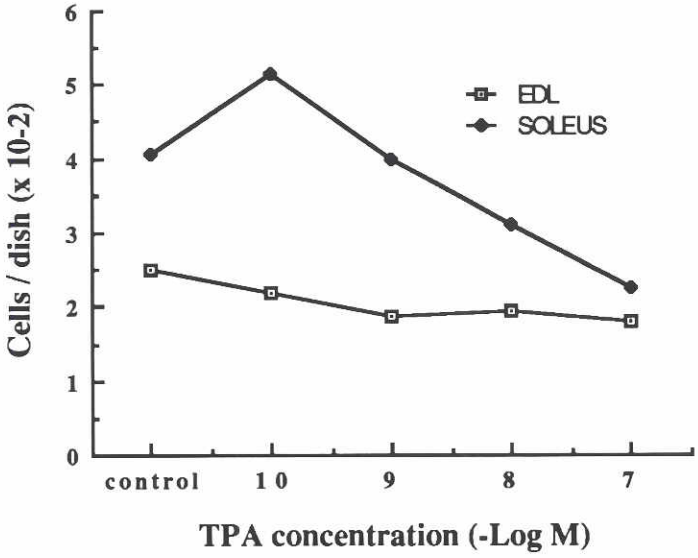


Fig.16. Effect of TPA treatment on the proliferation of satellite cells isolated from *Soleus* or EDL, after 5-day culture. *A low concentration (10^{-10}M) stimulateS proliferation in* Soleus *satellite cells; higher concentrations have inhibitory effects. Under our conditions, proliferation in EDL cells remained unchanged.*

fetal myoblasts, because the level of PKC activity did not change significantly in either cell type but important changes in subcellular PKC distribution occurred at fusion. These results suggested that the role of PKC might change with the stage of differentiation. They also implied that the differences described by Cossu's group between the sensitivity to phorbol ester of myoblasts and satellite cells were not connected with the phorbol ester receptors but might be caused by events subsequent to PKC activation.

Satellite cells cultured* in vitro *seem to " remember " their origin

The above results showed that the satellite cells isolated from adult innervated muscles may differ from the fetal myoblasts obtained before muscle innervation. Other results, mentioned below, also showed that satellite cells should be considered as engaged in different pathways according to whether the muscle from which they were isolated was fast or slow.

The satellite cells that we isolated from slow *Soleus* and fast EDL muscles differed in their density, myogenicity and response to t-PA treatment. Cell density is known to be greater in *Soleus* than in EDL (Gibson and Schultz, 1983) and we therefore obtained more satellite cells from *Soleus* (about 600 cells/mg of fresh weight vs 300 for EDL). After 4 days the density of cultures plated in multiwell dishes with the same number of cells (10^4 cell/well) rose 2.5 times higher in *Soleus*. After 8 days, the number of myotubes formed per well was 3.5 times larger for *Soleus* than EDL (68 vs 20), and the mean numbers of nuclei per myotube for each muscle type were 10 and 6 respectively. As mentioned above larger amounts of both plasminogen activators, UK and t-PA, were present in slow *Soleus* muscles and their satellite cells cultured *in vitro* (Barlovatz-Meimon *et al.*, 1990). We concluded that the myogenicity of satellite cells was greater in slow than in fast twitch muscles. The satellite cells originating from these muscles also differed in their sensitivity to TPA treatment. Thus, in cultures of satellite cells from *Soleus*, 5 days of treatment with high TPA concentrations of 10^{-7} or 10^{-8} once a day inhibited mitosis and myogenesis but treatment with a low TPA concentration (10^{-10}M) significantly stimulated these processes. In EDL, on the other hand, satellite cell proliferation remained unchanged in the presence of TPA whatever the concentration used (Fig.16). The possibility that the difference between these responses was due to a difference in the number of TPA receptors, or in the responses to PKC, is now under investigation. These results emphasized that the origin of satellite cells is of crucial importance when studying factors affecting proliferation and differentiation processes.

Concluding remarks

In pregnant rats, myotubes differentiate into muscle fibers at 17-18 days, during the perinatal period. Innervation develops at that time and typing of myofibers takes place leading to the formation of slow, fast and fast/slow twitch muscles; these fibers are accompanied by myogenic cells derived from myoblasts which are known as satellite cells. As a tentative conclusion, we propose that these satellite cells no longer remain in the undifferentiated state of myoblasts, but are engaged in a further differentiation pathway, and in particular express the characters of the myofibers they accompanied. Whether the differences between the characters of satellite cells from fast and slow muscles are permanent is not known, nor do we know how they are induced. However, the latest results given above justify the use of true myogenic satellite cells in our experiments, in order to ensure that our *in vitro* conditions are the nearest possible to those of normal regeneration, which it is our main purpose to study.

Acknowledgments

This work was supported by the Association des Myopathes de France, by INSERM grant N° 876015 and the ATP Franco-Polonaise 2C15.

References

ABRAHAM, J.A., MERGIA, A., WHANG, J.L., TUMOLO, A., FRIEDMAN, J., HJERRILD, K.A., GOSPODAROWICZ, D. and FIDDES, J. C. (1986). Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science 233:* 545-547.

ALLEN, R.E., DODSON, M.V., LUITEN, L.S. and BOXHORN, L.K. (1985). A serum-free medium that supports the growth of cultured skeletal muscle satellite cells. *In Vitro 21:* 636-640.

ALLEN, R.E., Mc ALLISTER, P.K. and MASAK, K.C. (1980). Myogenic potential of satellite cells in skeletal muscle of old rats. *Mech. Ageing Dev. 13:* 105-113.

ALTERIO, J., COURTOIS, Y., ROBELIN, J., BECHET, D. and MARTELLY, I. (1990). Acidic and basic fibroblast growth factor mRNA are expressed by skeletal muscle satellite cells. *bioch. Biophys. Res. Commun. 166*: 1205-1212.

ALTERIO, J., HALLEY, C., BROU, C., SOUSSI, T., COURTOIS, Y. and LAURENT, M. (1988). Characterization of bovine acidic FGF cDNA clone and its expression in brain and retina. *FEBS Lett. 242:* 41-46.

ASKANAS, V. and ENGEL, W. (1975). A new program for investigating adult human skeletal muscle grown aneurally in tissue culture. *Neurology. 25:* 58-67.

BARLOVATZ-MEIMON, G., FRISDAL, E., BASSAGLIA, Y., HANTAI, D., ANGLÉS-CANO, E. and GAUTRON, J. (1990). Relationship between plasminogen activators and regeneration capacities of rat skeletal muscles. In *Regulation of Extravascular Fibrinolysis in Neurologic System Development and Diseases* (Ed. B.W. Festoff) NATO Advanced Studies Series. Series A: Life Sciences. Plenum Press, New York.

BISCHOFF, R. (1974). Enzymatic liberation of myogenic cells from adult rat muscle. *Anat. Rec.180:* 645-662.

BISCHOFF, R. (1986). Proliferation of muscle satellite cells on intact myofibers in culture. *Dev. Biol. 115:* 129-139.

BISCHOFF, R. and HOLTZER, H.(1969). Mitosis and the process of differentiation of myogenic cells *in vitro*. *J. Cell Biol. 41:* 188-200.

BOUCAUT, J.C. and DARRIBERE,T. (1983). Fibronectin in early amphibian embryos. *Cell Tissue Res. 234:* 135-145.

BRUGAL, G. and CHASSERY, J.M. (1971). Un nouveau système d'analyse densitométrique et morphologique des préparations microscopiques. *Histochemistry 52:* 241-258.

CAMPION, D. R. (1983). The muscle satellite cell: a review. *Int. Rev. Cytol. 87:* 225-250.

CARLSON, B.M. (1973). The regeneration of skeletal muscle: a review. *Am. J. Anat. 137:* 119-150.

CASTAGNA, M., TAKAI, Y., KAIBUCHI, K., SANO, K., KIKKAWA, U. and NISHIZUKA, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor promoting phorbol esters. *J. Biol. Chem. 257:* 7847-7851.

CHIQUET, M., EPPENBERG, H.M. and TURNER, D. (1981). Muscle morphogenesis: evidence for an organizing function of exogenous fibronectin. *Dev. Biol. 88:* 220-235.

CHIQUET, M., PURI, E. and TURNER, D. (1979). Fibronectin mediated attachment of chicken myoblasts to a gelatin coated substratum. *J. Biol. Chem. 254:* 5475-5482.

COSSU, G., MOLINARO, M. and PACIFINI, M. (1983). Differential response of satellite cells and embryonic myoblasts to a tumor promotor. *Dev. Biol. 98:* 520-524.

COSSU, G., CICINELLI, P., FIERI, C., COLETTA, M. and MOLINARO, M. (1985). Emergence of TPA-resistant cells during muscle histogenesis of human limb. *Exp. Cell Res. 160:* 403-411.

DANØ, K., ANDREASEN, P.A., GRØNDAL-HANSE, J., KRISTENSEN, P., NIELSEN, L.S. and SKRIVER, L. (1985). Plasminogen activators, tissue degradation and cancer. *Adv.Cancer Res.44:* 139-266.

DI MARIO, J., BUFFINGER, N., YAMADA, S. and STROHMAN, R.C. (1989). Fibroblast growth factor in the extracellular matrix of dystrophic (mxd) mouse muscle. *Science 244:* 688-690.

EHRISTMANN, R., CHIQUET, M. and TURNER, D.C. (1981). Mode of action of fibronectin in promoting chicken myoblast attachment. *J. Biol. Chem. 256:* 4056-4062.

FESTOFF, B.W., HANTAI, D., SORIA, C., SORIA, J., FARDEAU, M. (1986). Plasminogen activator (PA) in muscle, its activation post denervation. In *Molecular Aspects of Neurobiology* (Ed. R. Levi-Montalcini). Springer Verlag, Berlin, pp. 162-166.

FLORINI, J.R. and MAGRI, K.A. (1989). Effects of growth factors on myogenic differentiation. *Am. J. Physiol. 256:* 701-711.

FRANQUINET, R. (1988). *In vitro* proliferation and differentiation of larval and adult urodele myogenic cells. In *Control of Cell Proliferation and Differentiation during Regeneration* (Ed. J. Anton). Monographs in Developmental Biology, Karger (Basel), Vol. 21, pp. 70-77.

FRANQUINET, R. (1989). *In vitro* potentialities of myogenic cells isolated from adult urodele muscles. In *Recent Trends in Regeneration Research* (Eds. V. Kiortsis, V. Koussoulakos and H. Wallace). NATO Advanced Studies Series. Series A: Life Sciences, Vol. 172. Plenum Press, New York, pp.173-174.

FRANQUINET, R., AAMIRI, A., GAUTRON, J. and LE MOIGNE, A. (1988). *In vitro* proliferation and differentiation of myogenic cells from adult *Xenopus*. *Biol. Struct. Morphog. 1:* 84-88.

GARDNER, J.M. and FAMBROUGH, D.M. (1983). Fibronectin expression during myogenesis. *J. Cell Biol. 96:* 474-485.

GAUTRON, J. (1988). Culture de cellules musculaires squelettiques. In *Cultures de cellules animales*. (Eds. M. Adolphe and G. Barlovatz-Meimon). INSERM, Paris, pp.231-243.

GIBSON, M.C. and SCHULTZ, E. (1983). Age-related differences in absolute number of skeletal muscle satellite cells. *Muscle Nerve 6:* 574-580.

GULATI, A.K., REDDI, A.H. and ZALEWSKI, A.A. (1983). Changes in the basement membrane zone components during skeletal muscle fiber degeneration and regeneration. *J. Cell Biol. 97:* 957-962.

HANTAI, D., TASSIN, A.M., GAUTRON, J. and LABAT-ROBERT, J. (1985). Biosynthesis of laminin and fibronectin by rat satellite cells during myogenesis *in vitro*. *Cell. Biol Int. Rep. 9/7:* 647-654.

HAUSCHKA, S.D. (1966). The influence of collagen on the development of muscle colonies. *Proc. Natl. Acad. Sci. USA 55:* 119-126.

HAY, E.D. (1968). Dedifferentiation and metaplasia in vertebrate and invertebrate regeneration. In *The Stability of the Differentiated State* (Ed. H. Ursprung). Springer Verlag, Berlin, pp. 85-108.

HAY, E.D. and DOYLE, C.M. (1979). Absence of reserve cells (satellite cells) in non-regenerating muscles of mature newt limbs. *Anat. Rec. 175:* 339-340.

KINTNER, C.R. and BROCKES, J.P. (1984). Monoclonal antibodies identify blastema cells derived from dedifferentiating muscle in newt limb regeneration. *Nature 308:* 67-69.

KONIGSBERG, U.R., LIPTON, B.H. and KONIGSBERG, I.R. (1975). The regeneration response of single mature muscle fibers isolated *in vitro*. *Dev. Biol. 45:* 260-275.

LASSALLE, B., GAUTRON, J., MARTELLY, I. and LE MOIGNE, A. (1989). Image analysis of rat satellite cell proliferation *in vitro*. *Cytotechnology 2:* 213-224.

LATHROP, B., OLSON, E. and GLASER, L. (1985a). Control by fibroblast growth factor of differentiation in the BC3 H1 muscle cell line. *J. Cell Biol. 100:* 1540-1547.

LATHROP, B., THOMAS, K. and GLASER, L. (1985b). Control of myogenic differentiation by fibroblast growth factor is mediated by position in the G1 phase of the cell cycle. *J. Cell Biol. 101:* 2194-2198.

LE MOIGNE, A., LASSALLE, B., MARTELLY, I. and GAUTRON, J. (1989). Image analysis of proliferation and fusion in cultured satellite cells. In *Recent Trends in Regeneration Research*. (Eds. V. Kiortsis, S. Koussoulakos and H. Wallace). NATO Advanced Studies Series. Series A: Life Sciences, Vol. 172. Plenum Press, New York, pp. 159-168.

LENTZ, L. (1969). Cytological studies of muscle dedifferentiation and differentiation during limb regeneration of the newt *Triturus*. *Am. J. Anat. 124:* 447-480.

LIPTON, B.H. and SCHULTZ, E. (1979). Development fate of skeletal muscle cells. *Science 205:* 1292-1294.

LOUGH, J. and BISCHOFF, R. (1979). Differentiation of creatine phosphokinase during myogenesis: quantitative fraction of isoenzyme. *Dev. Biol. 57:* 330-344.

MARTELLY, I., GAUTRON, J. and MORACZEWSKI, J. (1989). Protein kinase C activity and Phorbol ester binding to rat myogenic cells during growth and differentiation. *Exp. Cell Res. 183:* 92-100.

MAURO, A. (1961). Satellite cells of skeletal muscle fibers. *J. Biophys. Biochem. Cytol. 9:* 493-495.

MOSS, F. P. and LEBLOND, C.P. (1971). Satellite cells as the source of nuclei in muscles of growing rats. *Anat. Rec. 170:* 421-436.

MUNAIM, S.I., KLAGSBURN, M. and TOOLE, B.P. (1988). Developmental changes in fibroblast growth factor in the chicken embryo limb bud. *Proc. Nat. Acad. Sci. USA 85:* 8091-8093.

NIEDEL, J.E., KULIN, L.J. and VANDERBARK, G.R. (1983). Phorbol diester receptor copurifies with protein kinase C. *Proc. Natl. Acad. Sci. USA 80:* 36-40.

PLAGHI, L. (1985). Régénération et myogénèse du muscle strié. *J. Physiol. 80:* 51-110.

POPIELA, H. (1976). Muscle satellite cells in *Urodele* amphibians: facilitated identification of satellite cells using ruthenium red staining. *J. Exp. Zool. 198:* 57-64.

POUCHELET, M. and BARLOVATZ-MEIMON, G. (1989). *A story of Fusion: Myogenic Cells in Culture* (Microcinematographic Film). INSERM Doc. N° SERM 809062001 to 89071002. Service Audiovisuel de l'INSERM, Le Vésinet.

ROSARIO, R.A., DELOUVEE, A., YAMADA, K.M., TIMPL, R. and THIERY, J.P. (1983). Neural crest cell migration: requirement for exogenous fibronectin and high cell density. *J. Cell Biol. 96:* 462-473.

SCHRAG, J.A. and CAMERON, J.A. (1983). Regeneration of adult newt skeletal muscle tissue *in vitro*. *J. Embryol. Exp. Morphol. 77:* 255-271.

THIERY, J.P., DUBAND, J.L. and DELOUVEE, A. (1982). Pathways and mechanisms of avian trunk neural crest cell migration and localization. *Dev. Biol. 93:* 324-343.