The four populations of myoblasts involved in human limb muscle formation are present from the onset of primary myotube formation

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SUMMARY

To understand how and when myogenic precursor cells become committed to their particular developmental programs, we have analysed the different populations of myoblasts which grow out from explants of muscle tissue isolated from human limb buds from the beginning of primary fibre formation throughout subsequent development and post-natal growth.

Four phenotypically distinct types of myoblasts were identified on the basis of their expression of desmin, myogenin and myosin heavy chain isoforms (MyHC), and after 5 and 20 divisions, cells were cloned. All four types of myoblasts were present at the beginning of primary myogenesis. Each respective phenotype was stably heritable through cloning and subsequent proliferation.

The type 1 clones correspond to a novel class of myoblasts never described during human development, that biochemically differentiates, but does not fuse. Type 2 clones are composed of small myotubes expressing only embryonic MyHC. Type 3 clones are composed of thin and long myotubes expressing both embryonic and fetal MyHCs. The type 4 clones are composed of myotubes that have a phenotype very similar to human satellite cells.

Contrasting with others species, no other population of myoblasts appear during fetal development and only the relative number of these four types changes.

Key words: Human, Skeletal muscle, Myoblast, Cell lineage

INTRODUCTION

Vertebrate skeletal muscle formation can be divided into two distinct phases (primary and secondary myogenesis) based upon the temporal appearance of the myotubes within the individual muscles and upon the histology of the fibres and surrounding cells (Kelly and Zacks, 1969; Ontell and Kozeka, 1984; Ross et al., 1987; Harris et al., 1989; Duxson et al., 1989).

Primary muscle fibres, which are formed first, can be distinguished from the secondary fibres by their larger size and by the expression of muscle specific proteins such as myosin heavy chain isoforms (MyHC). In both rodents and humans, multiple MyHC isoforms have been identified and are expressed in a developmental and fibre type specific manner (Narusawa et al., 1987; Butler-Browne et al., 1990; Lyons et al., 1990; Condon et al., 1990a; Barbet et al., 1991) and can be used to identify primary and secondary generation myotubes. For example, in human quadriceps, the primary fibres, which form between 6 and 8 weeks of development, express embryonic, fetal and slow myosin heavy chains (Butler-Browne et al., 1990; Barbet et al., 1991; Hauschka, 1994; Tanaka et al., 1995) whereas the secondary fibres, which form

between 8 and 18 weeks of development (Barbet et al., 1991) are smaller and initially express embryonic, fetal and fast isoforms but not slow MyHC. As these secondary fibres mature the developmental myosins are gradually replaced by either adult slow or fast isoforms as the mature pattern of motor innervation becomes established.

Using the expression of fast and slow MyHCs to follow muscle development, it has been clearly demonstrated in both birds (Fredette and Landmesser, 1991) and rodents (Condon et al., 1990b; Hughes and Ontell, 1992) that fibre type formation and fibre type diversity proceeds normally in the absence of innervation but further development and maturation of the muscle will be profoundly impaired. This would be consistent with the hypothesis that the initial diversity which is seen in the muscle fibres as they form is due to the existence of different populations of myoblasts with distinct myosin heavy chain phenotypes. During muscle development, myoblasts can be isolated and cultivated in vitro. These myoblasts form myotubes which are capable of expressing different MyHC isoforms in the absence of innervation. By using antibodies specific for the different isoforms of MyHC, it has been demonstrated in both birds (Miller and Stockdale, 1986a,b; Stockdale and Miller, 1987; Mouly et al., 1987; Stockdale,

1992) and rodents (Vivarelli et al., 1988; Smith and Miller, 1992) that distinct types of myoblasts appear sequentially during skeletal muscle development.

The results obtained during human muscle development have not been so clear. In the human limb at 7 weeks of development, Hauschka (1974a) identified three distinct classes of human myoblasts (A, B and C) based upon their clonal morphology and extent of fusion. However, in a more recent study, Cho et al. (1993) looked at the biochemical phenotype of human myoblasts at the same stage of development but did not reveal such a diversity. These authors showed that myoblasts isolated at 7 weeks of development fused to form myotubes which coexpressed a slow myosin heavy chain identified by the antibody A4951 and an embryonic myosin heavy chain.

In this study we have combined both molecular and morphological techniques to identify and characterise the different classes of myoblasts that are involved in the formation and growth of human limb muscles. The different populations of myoblasts were isolated from explants of human limb buds from the beginning of primary fibre formation throughout subsequent development and post-natal fibre growth. The populations of cells obtained were cloned after 5-20 divisions.

By analysing the myoblast populations which occur from primary myogenesis onward we found four distinct myoblasts types (type 1, 2, 3 and 4) whose relative numbers changed during subsequent developmental stages. Previous studies by the group of Hauschka (Hauschka, 1974a; White et al., 1975; Seed and Hauschka, 1984) had shown that an early type of colony forming myoblast clearly preceded the appearance of later types i.e. even before primary myogenesis is triggered.

However, in this study, we have demonstrated that all four types of myoblast are present at as early as 7 weeks of development, a stage at which primary myotubes were just beginning to form in vivo. The type 1 myoblasts, which are very numerous at 7 weeks of development, present a novel type of clone since these cells express myogenin and embryonic MyHC but never fuse to form multinucleated structures in vitro. During primary and secondary myogenesis there is a progressive decrease in the number of type 1, 2 and 3 clones and an increase in myoblasts which form type 4 colonies, these type 4 cells have a phenotype which is identical to that previously described (Edom et al., 1994) for adult satellite cells.

MATERIALS AND METHODS

Isolation and culture of human myoblasts

The isolation and culture of human muscle cells was carried out in full agreement with the French legislation on ethical rules. Muscle biopsies were taken under sterile conditions during autopsy from the legs of embryos (3 embryos at 7, 2 embryos at 8.5 and 1 embryo at 9 weeks of development), from the quadriceps of fetuses (2 fetuses at 14, 2 fetuses at 18 and 2 fetuses at 24 weeks of development) and from 2 new-born infants (38 weeks of development). It should be noted that at the two earliest stages used in this study, biopsies were taken in the proximo-ventral region of the limb bud which will give rise to the quadriceps, not yet individualised at these early stages. The embryos, stored at 4 degrees in DMEM (Gibco) without serum, were dissected within 24 hours following voluntary abortions. The muscle biopsies showed no signs of neuromuscular disease.

The muscle was finely minced and 25-50 explants were plated into non-coated Petri dishes (diameter 60 mm) in 2 ml of growth medium consisting of DMEM containing 0.1 g/l gentamicin and 20% fetal calf serum (Gibco). Serum was selected to optimize the growth capacity of human myoblasts. Although differentiation was induced in the absence of serum, the differentiation capacity of human myoblasts after cultivation in that serum was also included in the test. Human muscle cells were cultivated at 37°C in a humid atmosphere containing 7% CO_2. Once mononucleated cells had migrated out from the explants (between 2 or 3 days), the cells were removed by trypsinization (1.5% trypsin, 0.04% EDTA) and replated at $1.8{\times}10^3$ cells/cm^2 in 5 ml of the same growth medium in 60 mm dishes. This culture medium was changed three times a week. At the time of cell isolation, all cell populations were considered to be at 1 mean population doubling. The number of population doublings at every passage was calculated as n/ln2 where n is the number of cells at the time of passage divided by the number of cells initially attached after seeding.

Clonal analysis

Cells were expanded (an average of one passage every 3 days). Between 5 and 20 divisions after their isolation, they were plated at clonal density (6 cells/cm^2). Under these conditions, there is no overlapping of individual clones. When individual clones contained about 100-200 cells, growth medium was replaced by differentiation medium. For all experiments, a minimum of 100 clones were counted. In parallel experiments, individual clones containing 100 cells were removed by trypsinization and were replated into four dishes (100 mm). At confluence (about 12 divisions), the medium was changed to induce cell differentiation.

Induction of differentiation

To induce differentiation, cells were plated onto collagen-coated dishes and growth medium was replaced, after the cultures were rinsed with DMEM, by 5 ml of DMEM without serum but supplemented with 10 µg/ml of insulin (Sigma) and 100 µg/ml of transferrin (Gibco) (Edom et al., 1994).

Differentiation capacity

The efficiency of differentiation was quantified by counting the number of nuclei in multinucleated myotubes as a percentage of the total number of nuclei. The ratio between these two values is defined as the percentage of fusion. At least 1000 nuclei were counted per dish.

Immunocytochemistry

Results presented in this study were obtained after 6 days in differentiation medium, however, the same results were obtained when cells were left for 14 days in differentiation medium. This is not surprising, since a complete set of MHC isoforms is already detected after 6 days.

The cultures were fixed with 95% ethanol for 10 minutes. Non specific binding sites were blocked for 30 minutes with non immune serum. Cells were incubated for 1 hour at 37°C with primary antibodies. Antibodies against embryonic (2B6, A. Kelly; Gambke and Rubinstein, 1984), fetal (WB-MHCn) and slow (WB-MHCs) (Ecob-Prince et al., 1989; Barbet et al., 1991; Edom et al., 1994) MyHCs were used. Specific antibody binding was revealed with peroxidase using the avidin-biotin system (Vectastain). Antibodies were used at a dilution of 1/50 (2B6) or 1/15 (WB-MHCn, WB-MHCs).

Double-labelling experiments were performed to determine the myogenicity of clones using antibodies against myogenin (Mouly et al., 1993) and against the intermediate filament protein desmin (D33, DAKO). Antibodies were used either pure (anti-myogenin) or diluted 1/10 (D33 anti-desmin). Specific binding was revealed using peroxidase for the first antibody and alkaline phosphatase for the

second. To determine the percentage of positive cells, 1000 cells were counted.

Separation of myosin heavy chains

Actomyosin was prepared from cell population pellets according to the method of Butler-Browne et al. (1990). Myosin heavy chain isoforms were separated on 5% SDS-polyacrylamide gels containing 30% glycerol (Carraro and Catani, 1983) for 23 hours at 120 V at 4°C. To identify the myosin isoforms expressed by cell cultures, a control sample containing slow, fast IIA, fast IIB and fetal MyHCs was always migrated in the same gel. Since this analysis was not strictly quantitative, the quantity of protein loaded per gel was calculated in order to optimize the resolution of the MHC isoforms.

RESULTS

The myogenic potential of cells isolated from human muscle at different developmental stages was determined by staining undifferentiated cultures with an antibody against desmin. At 7 weeks of development, approximately 50% (minimum value: 43%; maximum value: 57%) of the cells which migrated out of the explants of human limb muscles expressed desmin. This number increased considerably during development so that by birth 95-98% of the cells were desmin positive.

The capacity of these desmin positive cells to differentiate was then determined by carrying out double labelling experiments using myogenin as a nuclear label for cell differentiation and these results were correlated with the percentage of fusion. At 7 weeks of development, 50% (minimum value: 43%; maximum value: 57%) of the myonuclei were found to express myogenin, whereas the maximum level of fusion obtained in these early cell cultures could reach 35%, but was frequently less (minimum value: 27%; maximum value: 35%). After 24 weeks of development, approximately 60% of the nuclei were myogenin positive (minimum value: 57%; maximum value: 60%) and the level of fusion of these cultures had increased and was usually between 60-65%.

From these results, it appears that, at early stages of development, in our culture conditions, a large number of the myogenic cells differentiate but do not fuse to form multinucleated myotubes whereas, at later time points (i.e. after 24 weeks of development), the majority of desmin positive/myogenin positive cells do fuse to form myotubes. In order to determine if these mononucleated differentiated myogenic cells were a distinct class of myoblast, we analysed the phenotype of clones of cells which were obtained from skeletal muscle at different stages of development.

A class of non fusing differentiated myoblast is present during early myogenesis in the human limb

A clonal analysis of cells obtained from muscle at 7 weeks of development revealed that, after 6 days in differentiation medium, the majority of the total clones did not fuse but remained mononucleated. Some of these clones had the elongated spindle shaped morphology characteristic of myoblasts (Fig. 1A).

Therefore, in order to determine if some of these non-fusing clones were myogenic, we looked at the expression of three myogenic markers, desmin, myogenin and embryonic MyHC. After 6 days in differentiation medium, 19% of the

non-fusing clones co-expressed both desmin and myogenin (Fig. 1B), and therefore were considered to be myogenic whereas the remaining clones (54.5%) expressed neither desmin nor myogenin and were considered to be non-
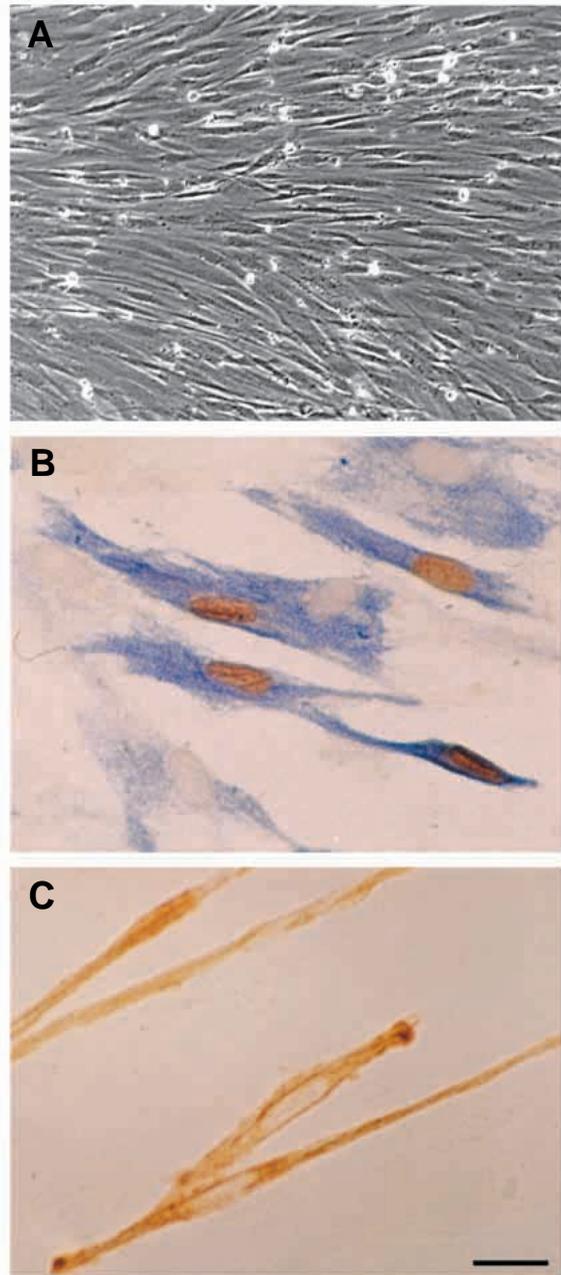
Fig. 1. Characterisation of mononucleated myogenic clones. Cells from human embryonic limbs (7 weeks of development) were cultivated in vitro and were analysed after 3 (B) or 6 days (A and C) in differentiation medium. (A) Phase contrast micrograph of type 1 clones. Note that no myotubes can be observed. Bar, 25 µm. (B) Double immunocytochemistry with antibodies against desmin and myogenin. Desmin was detected with alkaline phosphatase (blue) and myogenin with peroxidase (brown). As seen in this figure, these mononucleated cells co-express both desmin and myogenin confirming that they are both myogenic and differentiated. Bar 12, µm. (C) Immunocytochemistry with an antibody against embryonic MyHC. Specific binding was revealed with peroxidase. The type 1 cells differentiate and express embryonic MyHC. Bar, 12 µm.

Table 1. Different characteristics of the four types of myoblasts that are involved in the formation of human limb muscle

Types of myoblasts	Morphology of differentiated cells	Isoforms of MyHCs expressed	Number of nuclei per myotube
Type 1	Mononucleated cells	Embryonic	0
Type 2	Small myotubes containing 4-10 nuclei and without ramifications	Embryonic	4-10
Type 3	Long and thin myotubes containing more than 10 nuclei	Embryonic and fetal	10-50
Type 4	Large branched myotubes containing numerous nuclei (100 nuclei)	Embryonic fetal and slow	>100

myogenic. In addition to expressing desmin and myogenin all of the myogenic clones had a positive reactivity for embryonic MyHC, another marker of myogenic differentiation (Fig. 1C). None of the other MyHC isoforms were ever expressed by these mononucleated clones even after 6 days in differentiation conditions (data not shown). We have called the cells that form these differentiated non-fusing clones, type 1 myoblasts, and their relative number is indicated in Table 2.

Characterisation of three different classes of fusing myogenic cells already present at 7 weeks of development

At 7 weeks of development, in addition to mononucleated clones of non myogenic cells and type 1 myoblasts, three morphologically and biochemically distinct fused clones were characterised. We have called these types 2, 3 and 4 (see Table 1).

The type 2 clones were composed of small myotubes containing 4-10 nuclei surrounded by numerous mononucleated cells even in clonal conditions, where all cells are myogenic. The myotubes obtained after 6 or more days in differentiation conditions, were short and thin without any ramifications (Fig. 2A). All of the myotubes and some of the mononucleated cells reacted homogeneously with the embryonic MyHC antibody (Fig. 3). The immunocytochemical

reaction with antibodies against the other MyHC isoforms were negative both in myotubes and in mononucleated cells confirming that neither fetal nor slow MyHCs were expressed in type 2 clones (Fig. 3).

The type 3 clones were composed of thin myotubes much longer and a little larger than the type 2 clones, containing 10 or more nuclei. As for type 2 clones, these myotubes were never branched (Fig. 2B) and expressed embryonic MyHC as soon as they differentiated (Fig. 3). Interestingly, they also express fetal MyHC, which was detectable after 3 days in differentiation medium (Fig. 3). The reaction with the fetal antibody was less strong than with the embryonic antibody and was never observed in 100% of the myotubes. Reactivity with the slow antibody was never observed in any of these clones (Fig. 3).

The type 4 clones were composed of myotubes morphologically very different from the three other myogenic clones. The myotubes were very large with many ramifications and they contained numerous nuclei which were often in large groups rather than being aligned as in the type 3 clones (Fig. 2C). The percentage of fusion of these clones was very high, 70% (minimum value: 66%; maximum value: 74%). The type 4 clones all had the same pattern of expression of MyHCs: the myotubes were strongly reactive with embryonic, less strongly and more homogeneous with the fetal and had a patchy expression of the slow MyHC (Fig. 3).

Table 2. Relative number of the four types of myoblasts detected at different stages of development

	Type 1	Type 2	Type 3	Type 4
7 weeks				
Number of clones: 209	19	7	26	9
Myogenic clones: 61	(31±4%)	(11.5±0.45%)	(42.5±3.5%)	(15±7%)
8.5 weeks				
Number of clones: 315	67	20	63	47
Myogenic clones: 197	(34±5%)	(10±1.5%)	(32±5%)	(24±5%)
14 weeks				
Number of clones: 264	44	9	32	61
Myogenic clones: 146	(30±1.5%)	(6±1.5%)	(22±2%)	(42±2%)
18 weeks				
Number of clones: 316	9	11	40	131
Myogenic clones: 191	(4.5±0.5%)	(6±1%)	(21±1.5%)	(68.5±2.5%)
38 weeks				
Number of clones: 300	0	1	2	282
Myogenic clones: 285		(0.3±0.3%)	(0.7±0.3%)	(99±3.5%)

A clonal analysis of the different types of myogenic cell present in the human limb from 7 to 38 weeks (birth) of development was carried out.

Four different types of myogenic clones were identified and the percentage of the total number of these clones is indicated in brackets, as well as the variability corresponding to the different experiments performed at each stage.

Note the data indicated as 8.5 weeks correspond in fact to 8.5 and 9 weeks which were pooled.
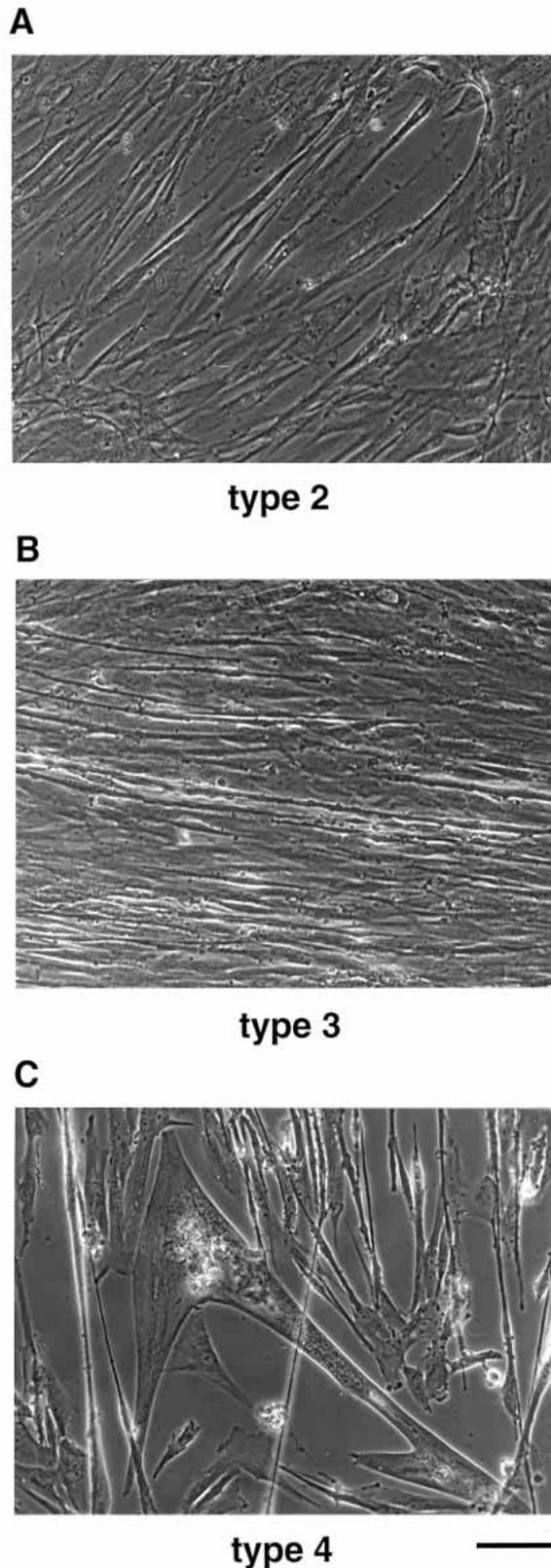
Fig. 2. Morphological characterisation of three types of fused clones isolated at 7 weeks of development. Phase contrast micrographs of type 2 (A), type 3 (B) and type 4 (C) clones after 6 days in differentiation medium. Type 2 myoblasts form very small myotubes, type 3 form longer myotubes and type 4, large branched myotubes. Bar, 25 μm.

Characterisation and quantification of the different types of myoblast implicated in the formation and growth of skeletal muscle

A similar clonal analysis was carried out on skeletal muscle at 8.5, 9, 14, 18 and 38 weeks of development. All of the clones which were obtained could be classified as either type 1, 2, 3, or 4 (Table 1). No additional type of myoblast was identified at any of these stages. However, the relative of number of each of these four types of myoblasts was found to vary depending upon the stage of muscle development. This is shown in Table 2. Between 7 and 14 weeks of development, there was a gradual decrease in the relative number of the type 1 myoblasts, and after 18 weeks of development type 1 clones were no longer detected.

Between 7 and 8.5 weeks of development, the number of both type 2 and type 3 myoblasts remained relatively stable (see Table 2). After this time, the number of these types of clones decreased progressively. No type 2 or 3 clones were detected in new-born muscle.

In contrast, throughout skeletal muscle development there was a progressive increase in the number of type 4 myoblasts. These cells were already present in the limb at 7 weeks of development and represent in our experiments 15% of the myogenic population. At 18 weeks of development (i.e. at the end of the formation of secondary fibres), type 4 myoblasts were predominant (68.5% of the myogenic clones). All of the myoblasts isolated after birth presented a type 4 phenotype. Since slow MyHC is only expressed by type 4 clones, the detection of this isoform was used to confirm biochemically, by gel electrophoresis, the gradual increase in the number of type 4 clones during development. As shown in Fig. 4, slow MyHC is first detected in cultures made from muscle at 8.5 weeks of development. The amount of this isoform then increases gradually during pre and postnatal development. It should be noted that slow MyHC was below the level of detection in early cultures at 7 weeks of development where the type 4 myoblasts represent only a very small percentage of the population.

DISCUSSION

The formation of skeletal muscle is characterised by the production of two populations of myotubes, primary and secondary. The way in which the primary and secondary fibres form in vivo has been the subject of extensive studies in birds, rodents and humans. The mechanism by which these primary and secondary fibres are generated has also been studied in vitro, by isolating the muscle precursors, i.e. myoblasts, and analysing their phenotype. Nutritional requirements, as well as the morphology of the myotubes formed in vitro, were the criteria used by Hauschka and his collaborators who defined distinct populations appearing successively during limb muscle development in the chicken (Bonner and Hauschka, 1974; White et al., 1975) as well as in humans (Hauschka, 1974a,b). Biochemical analysis confirmed this heterogeneity in the chick (Miller and Stockdale, 1986a,b), quail (Mouly et al., 1987), mouse (Smith and Miller, 1992) and human (Cho et al., 1993). Indeed, all of these studies showed that early and late myoblasts differ in the expression of several muscle specific proteins, such as MyHC isoforms. However, it has

Fig. 3. Pattern of MyHC expression in the different types of fused clones. Isolated clones were cultivated and analysed after 6 days in differentiation medium. Expression of embryonic, fetal and slow MyHCs was revealed by immunocytochemistry using antibodies against these different isoforms. Specific binding was detected with peroxidase using the avidin-biotin system (Vectastain). Type 2 clones express exclusively embryonic MyHC whereas type 3 clones co-express embryonic and fetal and type 4 clones co-express embryonic, fetal and slow MyHCs. Bar, 25 μm.
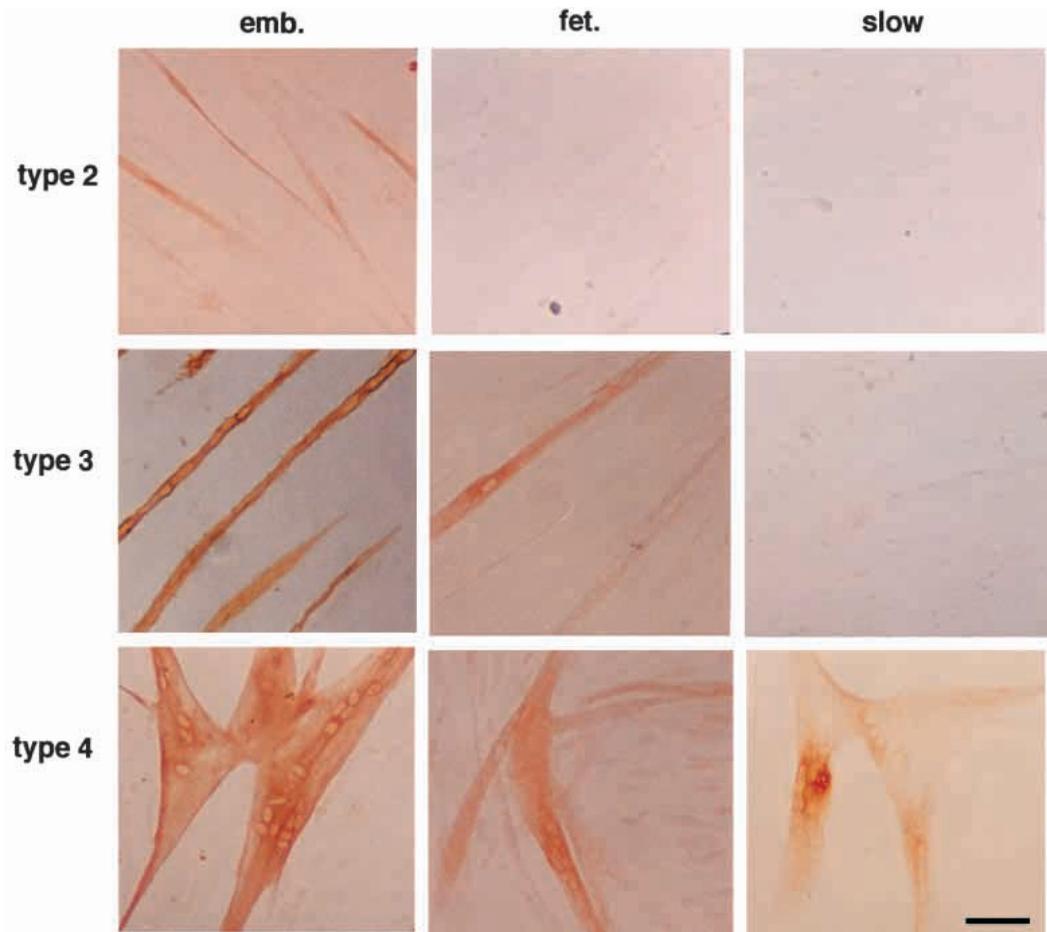
been shown that all limb muscle precursors originate from the somites (Chevallier et al., 1977; Christ et al., 1977; Jacob et al., 1978, 1979; Brand et al., 1985; Ordahl and Le Douarin, 1992) and migrate early into the limb bud during a short period of time (Mauger and Kieny, 1980; Seed and Hauschka, 1984; Van Swearinger and Lance-Jones, 1995). Therefore, all of the cells that are subsequently required to form skeletal muscle are present as soon as the first myoblasts begin to differentiate. In order to understand how and when myogenic precursor cells become committed to their particular developmental programs, we have analysed the different populations of myoblasts present in the human limb bud from the beginning steps of primary myotube formation until the end of muscle maturation.

We have described four different types of myoblasts isolated from explants all of which are present at the beginning of the formation of human primary fibers (Table 1). No other population of myogenic cells appears during fetal development and it is only the relative number of these four cell types that changes.

These results are similar to and extend the results obtained by Hauschka (1974a,b). As did Hauschka and his co-workers, we were able to characterise several populations of human myoblasts at 7 weeks of development, but in addition we have described a novel type of early myogenic cell, which we have called type 1. Moreover whereas Hauschka described a type of myoblast morphologically similar to type 4 cells only at 55

days of development (8 weeks of development) we had already detected this population at 7 weeks of development.

The dynamics of these different populations observed by Hauschka also differed. When Hauschka detect this last population called type D at 8 weeks of development, the two first populations of myoblasts had already disappeared and only the two last types of myogenic cells could be detected. In our study we were able to detect all four populations at 7
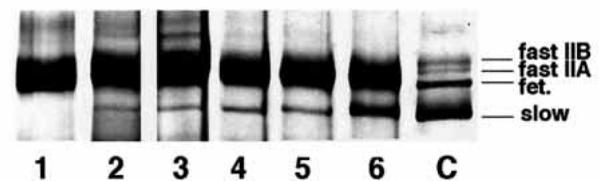
Fig. 4. Analysis of the MyHCs expressed by cultures at different stages of development. Slow MyHC can only be detected in cultures made from muscles after 8.5 weeks of development. This result confirms the absence of slow MyHC in the type 1, 2 and 3 clones. The amount of slow MyHC progressively increases with age. This is correlated with an increase in the percentage of type 4 clones with age. Lane 1, 7 weeks of development; lane 2, 8.5 weeks of development; lane 3, 14 weeks of development; lane 4, 26 weeks of development; lane 5, 38 weeks of development; lane 6, 5 months after birth; C, control sample).

weeks. The phenotype of these cells remained constant through cloning, which requires about 20 cell divisions.

Identification of a class of non fusing differentiated myoblasts in embryonic human limb muscles

In most of the previous studies, although myoblasts have been identified by their expression of desmin, cell fusion has been used as the criterion for studying their differentiation. In this study, we have examined the coexpression of desmin with myogenin, a myogenic regulatory factor which is known to be expressed in cell nuclei at the onset of cell differentiation, and embryonic myosin heavy chain, which is expressed in the cytoplasm of differentiated cells immediately after myogenin. We have been able to identify clones of differentiated mononucleated cells that in our conditions do not fuse to form multinucleated structures no matter how long they are left in differentiation conditions. These cells are committed to the myogenic pathway, as revealed by the expression of desmin (George-Weinstein et al., 1993) in the proliferating myoblasts. When the medium is changed, they differentiate and express both myogenin and embryonic myosin heavy chain. It was previously reported in the rat that cells giving rise to primary fibres do not initially express desmin in culture, but will after several passages (Kaufman et al., 1991; George-Weinstein et al., 1993). The detection of desmin in our experiments was performed after clonal growth, which represents probably many more divisions than the passages described by these authors. This could easily explain why such a transition was not observed in our human cultures.

Although we cannot exclude the possibility that these early cells could actually fuse in vivo in the embryonic environment or even under other culture conditions, these early clones represent a population of myogenic cell which are different from the other types of fusion competent myoblast which have been described previously (Hauschka, 1974a,b). Hauschka also reported non-fusing cells at that stage, but could not consider them as part of the myogenic compartment since no biochemical or immunological data were included at that time (Hauschka, 1974a). During early embryonic development in the mouse a similar type of non-fusing myogenic cell has also been described by the group of Cossu both in the somites and in the limb buds prior to the formation of the primary myotubes (Cusella-De Angelis et al., 1992). It was proposed that these mononuclear cells were equivalent to the pioneer cells that have been identified during invertebrate development (Jensen, 1990) which in insects, are the first cells to arrive in the limb bud and attach themselves to the adjacent ectoderm thus defining the limits for the migration of the subsequent populations of myoblasts (Ho et al., 1983; Jellies and Kristan, 1988; Bate, 1990). In the human limb at 7 weeks of development, we found that the type 1 myoblasts rather than being rare as one would expect for a pioneer type of cell were, in fact, one of the major class of cells and represented 31% of the myogenic clones that were isolated. This would suggest that type 1 myoblasts even if they are a type of pioneer cell would also have another function during development. We would suggest that type 1 myoblasts may therefore represent an intermediate cell type between the cells which have migrated from the somite and the myoblasts which fuse to form the primary fibres and would be involved in proliferating and forming the premuscle mass. However this is only a

speculation based on the limited capacity of these cells to fuse in our conditions. Another possibility would be simply that type 1 myoblasts represent in fact type A described by Hauschka (1974a,b), and that eventual differences in behaviour could be due to different culture conditions.

Are type 2 and 3 myoblasts the same as embryonic and fetal myoblasts?

In the mouse, Smith and Miller (1992) have shown that, before primary fibres are formed the vast majority of the mononucleated cells when grown in culture, do not synthesize fetal MyHC, whereas just before the secondary myotubes are formed, all of the myoblasts synthesize this isoform. These authors therefore hypothesised that the first type of myoblasts which they called embryonic, were responsible for forming the primary fibres and the second type of myoblast, which they called fetal, for forming the secondary fibres.

Type 2 and 3 myoblasts represent two distinct populations of myogenic cells which differ both in the morphology of the myotubes which they form and in the MyHC isoforms they express. The fact that our determination of the relative number of type 2 and type 3 cells shows no striking changes during primary myogenesis and then decreases progressively during the formation of secondary myotubes, would suggest that they are both implicated in the early stages of primary and secondary myogenesis. Our results are in direct agreement with those of Smith and Miller (1992) since our type 2 clones, which are also very similar to those other authors have described as embryonic myoblasts, express exclusively embryonic MyHC and our type 3 myoblasts express both embryonic and fetal MyHC and seem to be equivalent to what has been described as fetal myoblasts.

Similar results were obtained by Cho et al. (1993) who showed that human myoblasts that give rise to primary myofibres (i.e. isolated at 7 weeks of development) or secondary myofibres (i.e. isolated at 9 weeks of development) differ in their expression of embryonic and fetal MyHC isoforms. But, unlike Cho, we have been unable to detect slow MyHC either biochemically or immunologically; however, the conditions of differentiation in these two studies were different.

Type 4 myoblasts are responsible for the volumetric growth of skeletal muscle

Our results show that, whereas the number of type 1, 2 and 3 myoblasts decreases progressively during fetal development and disappears before birth, the number of type 4 clones increases progressively during development to become the predominant class present during secondary myogenesis and is the only type of myoblast detected after 24 weeks of development.

This is very similar to what has been described for birds and rodents where one class of myoblasts becomes the predominant species during fetal development (Cossu et al., 1983; Feldman and Stockdale, 1992; Hartley et al., 1992) with characteristics identical to the satellite cells described by Stockdale (1992). Moreover, Cossu and his collaborators were able to detect the presence of satellite cells in human muscle at as early as 15 weeks of development (i.e. during secondary myogenesis) by their sensitivity to phorbol esters (TPA) (Cossu et al., 1985). Using the method of clonal analysis, we were able to detect the presence of type 4 clones at 7 weeks of development just as

primary myogenesis begins. However, at 7 weeks, this is a minor population of myoblasts representing only 6.5% of the total cell (both myogenic and non-myogenic cells) population. It should be noted that type 4 clones have all of the characteristics that we have previously described (Edom et al., 1994) for adult myoblasts (satellite cells). We propose that these cells represent a pool of myoblasts that are implicated in the volumetric growth and lengthening of the primary and secondary generation fibres during development (Harris et al., 1989) and it is very interesting to note that they are present at the earliest stages of muscle formation.

Dynamic regulation of the different types of myoblasts during development

At the beginning of primary myotube formation, we were able to isolate four different types of myogenic clones with different morphological and biochemical phenotypes. Although signals for maturation might be absent in vitro, since the biochemical and morphological identity of these cells is maintained during subculturing, this would suggest that these four different types of myoblasts do not represent successive steps in the same lineage.

If one hypothesises that the mechanism of cellular migration described in birds (Mauger and Kieny, 1980; Seed and Hauschka, 1984; Van Swearinger and Lances-Jones, 1995) is similar in humans, and occurs over a relatively short period of time, then it would seem logical to suppose that at the beginning of muscle formation, all of the cells required for this process must be present in the limb. The results that we have obtained would support this hypothesis since we have demonstrated that four types of myoblasts which are involved in skeletal muscle formation are present prior to primary myogenesis. These results are also in agreement with the early clonal analyses carried out on both human and chick limb muscle by Hauschka and his coworkers (Bonner and Hauschka, 1974; Hauschka, 1974a,b; Seed et al., 1988; Hauschka, 1994).

It is interesting to note that one of the four classes described correspond to a novel population of human myoblast that is capable of differentiating without fusing in our conditions. This population is a good candidate for a primitive committed myoblast that will proliferate in the limb bud to form the premuscle masses. In addition we have shown that cells (type 4) with a similar phenotype to that which we have described previously for satellite cells (Edom et al., 1994) are also present at the early stage of human myogenesis. These cells could participate in the volumetric growth and lengthening of all fibers during development.

This study allows us to propose a model for myogenesis that integrates the results obtained by Hauschka and the idea that, even though several distinct populations of myoblasts exist, the different stages of skeletal muscle formation can depend on just one or more of these different populations.

Since all of the different types of myoblasts are present in the limb at the beginning of primary myogenesis, the different classes of myoblasts must respond to proliferate at the appropriate time in response to the different proteins and growth factors secreted by the surrounding cells and extracellular matrix. An example of such a mechanism has been proposed recently: a differential expression of protein kinase C between embryonic and fetal myoblasts allows cells to respond at the appropriate time to transforming growth factor β (Cusella-De Angelis, 1994; Zapelli et al., 1996). The model that we have proposed is consistent with the plasticity of the developing muscle tissue, a plasticity that cannot be explained only by the existence of successive classes of myoblasts, each one with a restricted program of differentiation.

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