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Both avian and mammalian embryonic myoblasts are intrinsically heterogeneous

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Summary

Adult skeletal muscles are composed of different fibre types. What initiates the distinctive muscle fibre type-specific specialization in a developing embryo is still controversial. *In vitro* studies of avian muscles have shown the expression of one of the slow myosin heavy chains, SM2, in only some myotubes. In this report we demonstrate the expression of another slow myosin heavy chain, SM1, restricted to only some chicken myotubes (presumptive slow) *in vitro*. We also demonstrate that as is the case for avian species, distinct fast and slow myogenic cells are detectable in mammalian species, human and rat, during *in vitro* development in the absence of innervation. While antibodies to fast myosin heavy chains stained all myotubes dark in these muscle cell cultures, antibodies to slow myosin heavy chains stained only a proportion of the myotubes (presumptive slow). The other myotubes were either unstained or only weakly stained with slow myosin heavy chain antibodies. The muscle cell cultures prepared from different developmental stages of rat skeletal muscles showed a reduction in the number of slow myosin heavy chain-positive myotubes with advancing foetal growth. It is concluded that embryonic myogenic cells that are likely to form distinct fast or slow muscle fibre types are intrinsically heterogeneous, not only in avian but also in mammalian species, although extrinsic factors reinforce and modify such commitment throughout subsequent development. © Kluwer Academic Publishers.

Introduction

Most adult skeletal muscles are composed of two main groups of fibre types, called slow and fast or type I and type II, that differ in their contractile or force-generating properties. These two main fibre types express different isoforms of a number of contractile and regulatory proteins of the myofibril (for reviews see Pette & Staron, 1990; Schiaffino & Reggiani, 1996) which endow these fibres with distinct functional properties. Myosin is one of the most abundant contractile proteins in the myofibril, and has been used extensively to study muscle development and differentiation. A myosin molecule is composed of two heavy and four light chains, with its ATPase activity located in the heavy chain subunit (Perry, 1996). It exists in several cell types and developmental stage-specific isoforms (Gauthier & Lowey, 1977; Whalen *et al*., 1981). The expression of myosin heavy chain isoforms can therefore be used both to distinguish muscle fibre types and to define the degree of muscle cell differentiation.

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The muscle fibre type-specific differences seen in the adult are not easily apparent during early embryonic or foetal development of many animals, but become clearer during later foetal and neonatal periods of growth. This has led to the common assumption that the failure to detect early differences in fibre types during embryonic development, and the gradual development of fibre type-specific differences during later foetal and neonatal development, is mainly related to the growing influence of extrinsic factors, such as innervation and thyroid hormones, and so on. The failure to detect early differences, however, may in part be caused by the high-level expression of developmental isoforms and only a low-level expression of cell type-specific isoforms of specialized myofibrillar proteins in early embryonic muscles. The delayed recognition of presumptive slow and fast muscle fibre types in most mammalian studies using conventional antibodies to myosin heavy chains has thus emphasized the role of extrinsic factors at the expense of intrinsic factors in determining the muscle fibre type characteristics. Such a hypothesis has been further supported by some recent *in vitro* studies of human skeletal muscles (Cho *et al*., 1993; Edom *et al*.,

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1994) that demonstrated the presence of both slow and fast muscle type myosin heavy chains in most myotubes, and hence a single *in vitro* myotube type when grown away from the *in vivo* interacting tissues. This differs from *in vitro* studies of avian embryonic muscles in which the presence of distinct slow and fast myotube types has been clearly demonstrated (Stockdale & Miller, 1987). Our present *in vitro* study identifies distinct fast and slow myotubes not only in avian but also in human and rat embryonic or foetal skeletal muscle cell cultures. This *in vitro* study also supports our earlier *in vivo* studies using a more specific antibody to a slow myosin heavy chain that detected the emergence of presumptive slow and fast myotubes at a much earlier stage, not only in the embryonic rodent limb (Dhoot, 1985, 1986) but also in somitic myotome (Dhoot, 1994a). Both *in vivo* and *in vitro* studies of avian as well as mammalian species thus show early myotubes to be phenotypically distinct and likely to form distinct fast or slow muscle fibre types, although they are subject to extensive modification by extrinsic factors throughout development, modifying their properties accordingly.

Materials and methods

Preparation of muscle cell cultures

Human muscle cell cultures were prepared from foetal quadriceps at 10–18 weeks of gestation. Muscle tissues were dissected out in calcium/magnesium-free phosphate buffered saline (PBS) and transferred to Ham's nutrient medium F10 (Life Technologies). Muscles were then cut into small fragments and digested in 2% trypsin (Sigma Chemical Co.) for 10 minutes at room temperature. The muscle suspension was triturated with a Pasteur pipette and the cell suspension collected in 15% foetal calf serum (FCS) in F10 medium. A single cell suspension was created by passing the suspension a few times through 19 gauge needle and by filtering it through a single layer of 0.45 μm Nitex filter. Cells were plated on gelatin-coated petri dishes. 25 μl of cell suspension containing approximately 1000 cells was spotted on to a 4 cm petri dish. Petri dishes with 2–3 spots each were incubated for 1 hour at 37°C with 5% CO_2, and then flooded gently with 1.5 ml of Dulbecco's modified Eagle's medium (DMEM) or Ham's F10 nutrient medium containing 15–20% FCS, 0.5% chick embryo extract and 1% penicillin, streptomycin and fungizone. The cell cultures were grown in this growth medium for 3–15 days after which time it was replaced with differentiation medium for 3–4 days. The differentiation medium was either F10 medium with 3% FCS, or DMEM medium with 10 $\mu g\ ml^{-1}$ insulin and 100 $\mu g\ ml^{-1}$ transferrin (Sigma Chemical Co.). Compared with human, muscle cell cultures from rat and chick myotome or foetal hindlimbs were grown for shorter periods.

Immunocytochemical procedure

For immunocytochemical staining, the muscle cell cultures growing on petri dishes were washed three times with PBS and fixed in 70% methanol for 10 minutes. The fixed cultures were either stained with antibodies or stored at −70°C until required. Cell cultures were stained with different antibodies, diluted in 10% FCS in PBS Tween, using immunoperoxidase or double immunofluorescence procedure. For double immunofluorescence, cell cultures were first stained with either slow or fast myosin heavy chain (MHC) antibodies using either FITC-labelled or TRITC-labelled secondary antibodies. After washing, the same cultures were then treated with the second primary antibody (fast if slow myosin heavy chain antibody was used in the first instance, but slow if fast myosin heavy chain was used first) which was then detected by the second fluorochrome-labelled antibody. After washing, treated sections were mounted in glycerol medium (Citifluor) and photographed using an Olympus microscope. For immunocytochemical analysis of muscle tissues, 6 μm thick sections were cut from adult or foetal muscle blocks frozen in liquid nitrogen. Serial sections were then stained by fast and slow myosin heavy chain antibodies using the immunoperoxidase procedure.

Specificities of antibodies

Antibody F2 was used to investigate the expression of fast myosin heavy chains (FMHC). This antibody reacts with both adult and developmental isoforms belonging to the fast class of myosin heavy chain, in avian as well as mammalian species (Williams & Dhoot, 1992). Only foetal myosin isoforms are usually present in all *in vitro* myotubes prepared from early foetal skeletal muscles and *in vivo* embryonic muscles. Small amounts of neonatal myosins may also be detected in longer term cultures or cultures prepared from late foetal and neonatal muscles (Smith & Miller, 1992). Since fast muscle-like foetal myosin isoforms are present in all myotubes during early *in vivo* and *in vitro* development, most FMHC antibodies usually do not enable the distinction between myotube types in early developing muscle. We therefore used a single FMHC antibody that detects the whole class of fast myosin heavy chains. The expression of another developmental isoform, namely ventricular myosin heavy chain (VMHC) present in avian myotubes, was detected using antibody 18 (Sweeney *et al*., 1989). Three different antibodies, 96J, M14 and 83, were used to investigate the expression of slow muscle type MHCs (Kilby & Dhoot, 1988). Antibody 83 recognizes both SM1 and SM2 myosin heavy chains in chicken (Williams *et al*., 1992), while antibody M14 recognizes only one of these MHCs, namely SM1 (Dhoot & d'Albis, 1993). Antibody 96J recognizes slow MHC in mammals as well as in chicken. In chicken, it does not react with SM2 MHC but shows a positive reaction with ventricular MHC (Williams *et al*., 1992).

Results

IDENTIFICATION OF PRESUMPTIVE SLOW AND FAST MYOTUBES DETERMINED BY THEIR MHC EXPRESSION PATTERN IN AVIAN EMBRYONIC CULTURED MYOTUBES

Before investigating the myoblast heterogeneity in mammals in which the expression pattern has been

more controversial, we decided to check our analytical approach in the avian system first, in which myoblast heterogeneity has been demonstrated in cell cultures prepared from embryonic muscles (Stockdale & Miller, 1987). When we prepared muscle cell cultures from early embryonic limb or somitic myotome, an antibody to slow myosin heavy chain that recognizes both SM1 and SM2 MHCs, (Williams *et al.*, 1992), stained a very small number of myotubes dark (Fig. 1a). Antibody M14 to SM1 MHC also stained only a few myotubes dark (Fig. 1c). Antibodies to FMHCs and VMHC, in contrast, stained virtually all myotubes dark at these stages (Fig. 1b, d, e). While both fast (Fig. 1b and e) and ventricular myosin heavy chain (Fig. 1d and f) antibodies stained all myotubes dark, the level of staining intensity for VMHC compared with FMHC, although equally intense, was more restricted within the myotubes.

IDENTIFICATION OF PRESUMPTIVE SLOW AND FAST MYOTUBES DETERMINED BY THEIR MHC EXPRESSION PATTERN IN *IN VITRO* AND *IN VIVO* MAMMALIAN MUSCLES

Human muscle cells

Most normal adult human skeletal muscles are composed of two main fibre types, type I and II, that usually express either slow (Fig. 2a) or fast myosin heavy chains (Fig. 2b). To determine myotube diversity in human foetal skeletal muscle, the myogenic cells for *in vitro* growth were prepared from 12–18 week human foetal quadriceps. Antibodies to FMHCs stained all myotubes equally dark in such muscle cell cultures (Fig. 2d). Antibodies to slow myosin heavy chain (Fig. 2c), in contrast, stained only a subset of the myotubes dark (e.g. myotubes labelled with long arrows), with the remaining myotubes being unstained (myotubes labelled with shorter arrows) or only weakly stained (myotubes labelled with hollow arrows).

Rat muscle cells

The presumptive slow and fast myotubes can be identified in rat foetal skeletal muscles using antibodies to slow myosin heavy chain (Fig. 3a, b). The distinction into these two fibre types has been reported to occur not only in the early embryonic limb muscles but also in the somitic myotome (Dhoot, 1994a).

When myogenic cells were isolated from early rat foetal limb or somitic myotome for *in vitro* growth, distinct slow and fast myotubes were identified using both immunoperoxidase and immunofluorescence procedures. As was the case for the human foetal and avian embryonic muscle cell cultures, antibodies to slow myosin heavy chain stained only some myotubes dark in muscle cell cultures prepared from early foetal limb (Fig. 3d and e) and somitic myotome (Fig. 3f and g). The remaining population of myotubes did not stain, while a small proportion stained weaker with variable intensity. Antibodies to FMHCs stained all myotubes dark, as expected.

To investigate whether the proportions of presumptive slow and fast myotube types changed during foetal development, the rat muscle cell cultures were prepared from somitic myotome and limb muscles from different foetal developmental stages. The proportion of slow myotubes in such cultures decreased with increasing foetal stage of muscle development (Fig. 4). For example, while nearly a third of the myotubes stained dark for slow MHC in muscle cell cultures prepared from 11 day foetal limb (Fig. 4a, b), this number was much reduced in myogenic cultures prepared from 13 day foetal limbs (Fig. 4c, d). None or very few myotubes stained dark for slow myosin heavy chain in muscle cell cultures prepared from limb muscles of foetuses older than 16 day gestation (Fig. 4e, f).

Discussion

Whether the distinct fast and slow muscle fibre type characteristics of adult muscles are intrinsically determined or develop gradually as a result of them being influenced entirely by extrinsic factors has remained controversial. Innervation clearly influences the muscle fibre phenotype, since changes in innervation type usually lead to changes in muscle fibre type in the adult (Buller *et al.*, 1960). Some fibre type characteristics, however, have been reported to emerge in the absence of innervation, although the proportions of fibre types in aneural muscles change drastically (Dhoot & Perry, 1982; Phillips *et al.*, 1986; Harris *et al.*, 1989; Condon *et al.*, 1990; Dhoot, 1994b). The studies of Hoh and Hughes (1988) have demonstrated that the expression of a jaw muscle specific myosin called superfast myosin in aneural jaw muscle regenerates in both their own and inappropriate limb muscle beds. Changes in the levels of thyroid hormones (Izumo *et al.*, 1986) and the degree of muscle stretch have also been shown to influence the fibre type composition (Loughna *et al.*, 1990). Regenerating avian myotubes express fibre type-specific differences during early stages of growth, but further fibre type-specific differentiation is dependent upon the presence of appropriate innervation (Kyprianou *et al.*, 1993; Madgwick *et al.*, 1995).

Both *in vitro* and *in vivo* studies of the emerging fibre type characteristics during development have been controversial. Part of this controversy has resulted from the differences observed in avian and

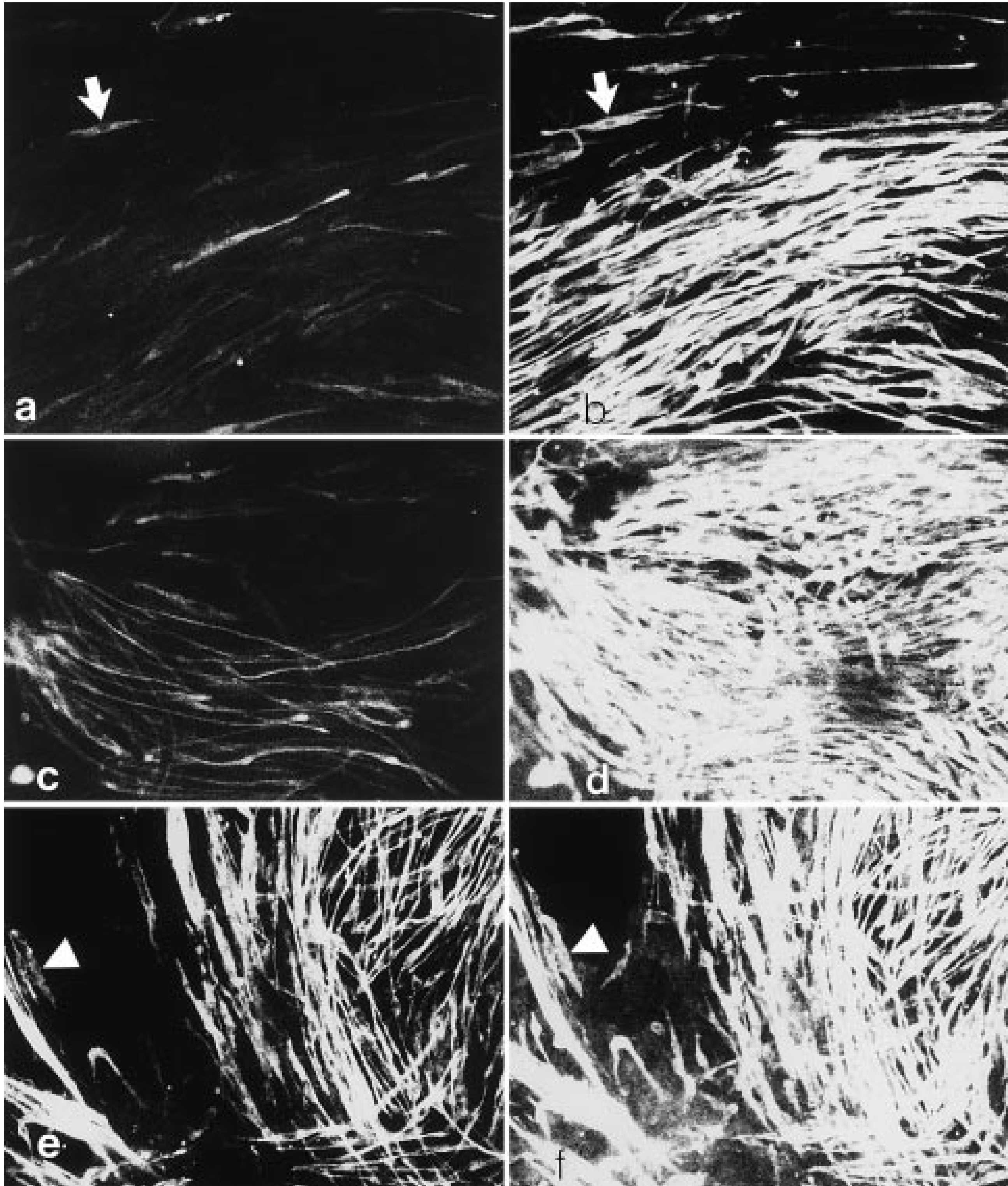


Fig. 1. Myotubes prepared from somitic myotome of 3.5 day chick embryos cultured for 3.5 days *in vitro* are stained with different myosin heavy chain antibodies using double immunofluorescence procedures. Myotubes in culture 1 in the top row (a, b), were stained with antibody 83 to slow myosin heavy chains SM1 and SM2 (a) and with antibody F2 to fast myosin heavy chains (b). Myotubes in culture 2 in the middle row (c, d) were stained with antibody M14 to SM1 (c) and antibody 18 to ventricular myosin heavy chain (d). Myotubes in culture 3 in the bottom lane (e, f) show comparative staining with antibodies 18 (e) and F2 (f). Similar arrows indicate the same areas in double-stained muscle cell cultures. Magnification × 225.

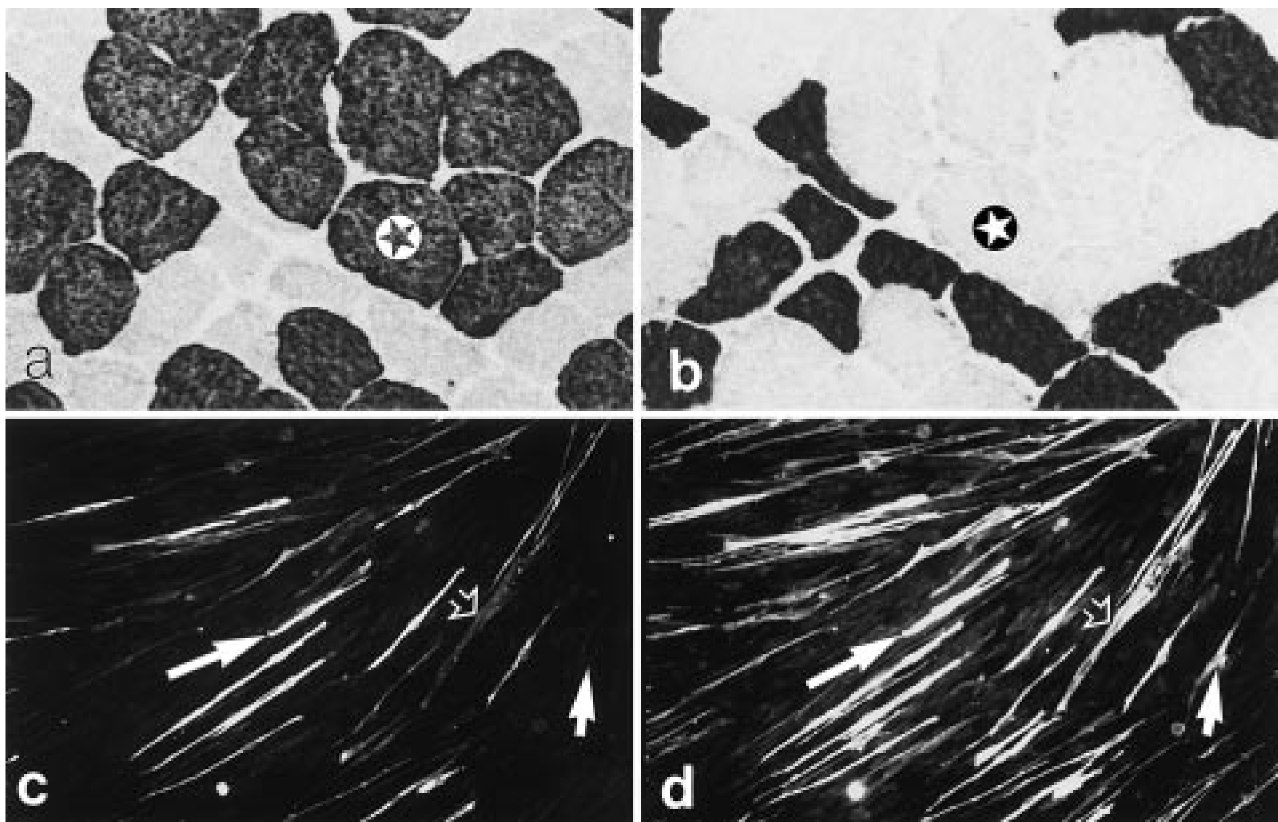
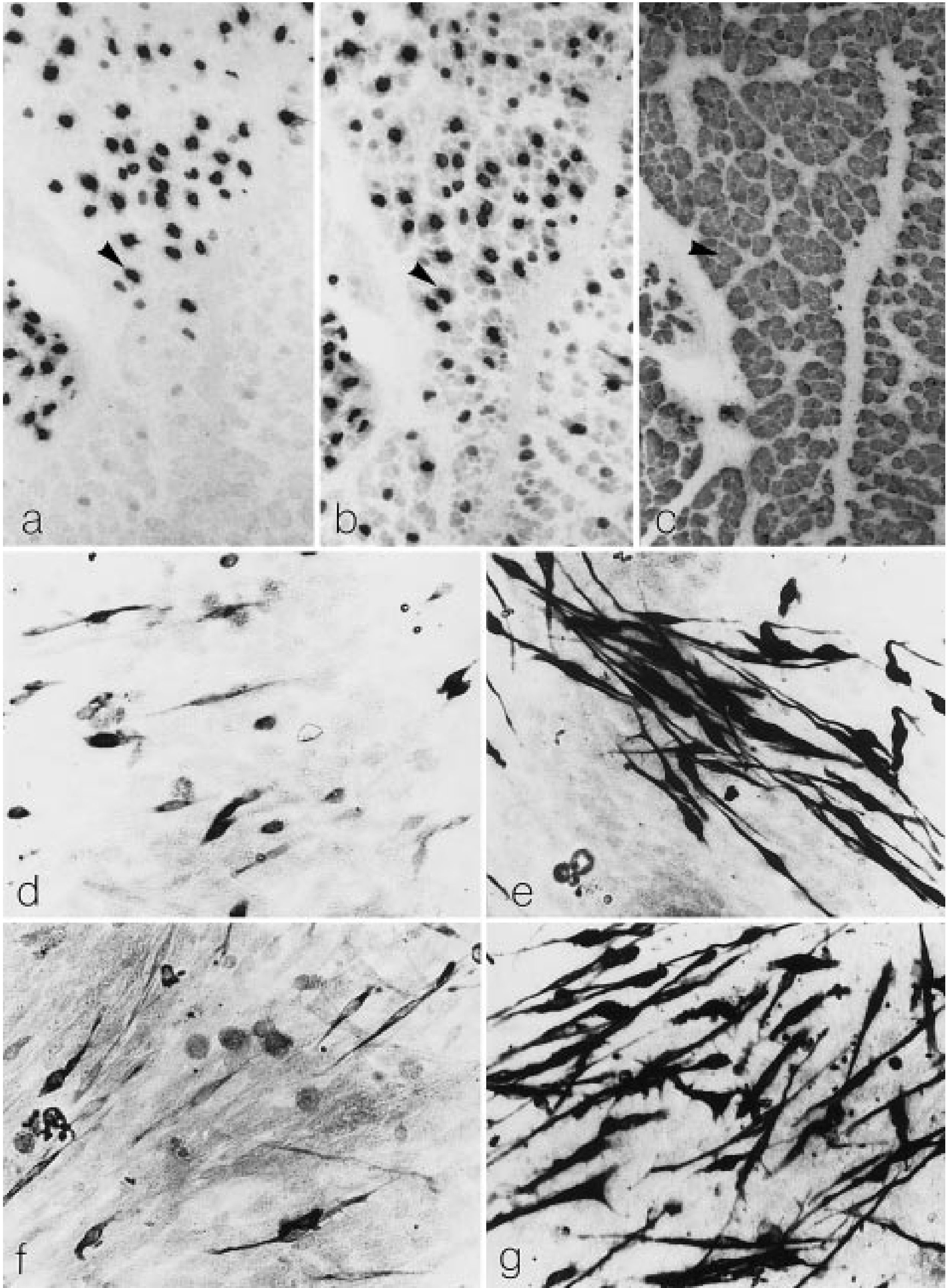


Fig. 2. Serial cross-sections of human biceps (a and b) and a muscle cell culture prepared from quadriceps muscle of 12 week human foetus after 21 days in culture (c and d), stained with antibody 96J to slow (a and c) and F2 to fast myosin heavy chains (b and d) by immunoperoxidase (a and b) and double immunofluorescence (c and d) procedures. Similar symbols or arrows indicate the same areas. Magnifications are ×202 (a, b) and ×124 (c, d).

mammalian muscles. While distinct slow and fast myotubes are identified in avian muscle cell cultures using some slow MHC-specific antibodies, *in vitro* mammalian myotubes, particularly human, have been reported to express both fast and slow myosin heavy chains in all myotubes. We investigated this problem further by *in vitro* studies of embryonic chicken as well as human and rat skeletal muscles. Our study of avian skeletal muscles showed the expression of slow MHC in only some myotubes when the muscle precursors for *in vitro* growth were isolated from somitic myotome or early embryonic limb muscles. The expression of both SM1 and SM2 MHC was restricted to only a small number of myotubes. This is in agreement with the SM2 MHC observations of Stockdale and Miller (1987), who reported the expression of this MHC in only a subset of the fibres. Our observations of the SM1 MHC expression pattern, using two antibodies (one SM1 MHC-specific and the other SM1 + SM2 MHC-specific), differ from avian studies in which SM1 MHC expression has been reported in all *in vitro* myotubes (Page *et al*., 1992). The present study supports our earlier *in vivo* investigation in which we observed the expression of not only SM2 but also SM1 MHC restricted to a small number of myotubes (Williams *et al*., 1992). All primary generation myotubes (Williams *et al*., 1992) and most *in vitro* myotubes in this study were observed to stain with another antibody called 96J, which in addition to SM1 MHC also recognizes ventricular MHC (Williams *et al*., 1992). It appears, therefore, that while ventricular and fast myosin heavy chains are present in virtually all *in vitro* myotubes, SM1 and SM2 MHC are restricted to only a small number of myotubes even during very early stages of myogenesis. Mashima and coworkers (1997) have similarly demonstrated the presence of slow troponin-T in only a subset of the avian *in vitro* myotubes.

Our *in vitro* studies of rat foetal skeletal muscles also demonstrated the presence of slow MHC in only a subset of the myotubes, further indicating the existence of distinct slow and fast myogenic cell lineages in developing embryonic muscles. The proportion of slow myotubes *in vitro* decreased with increasing foetal age of developing rat muscles used to prepare these cultures. For example, while a large number of slow MHC-positive myotubes were detected in muscle cell cultures prepared from embryonic or early foetal skeletal muscles, none or few slow MHC-positive myotubes were detected in cultures prepared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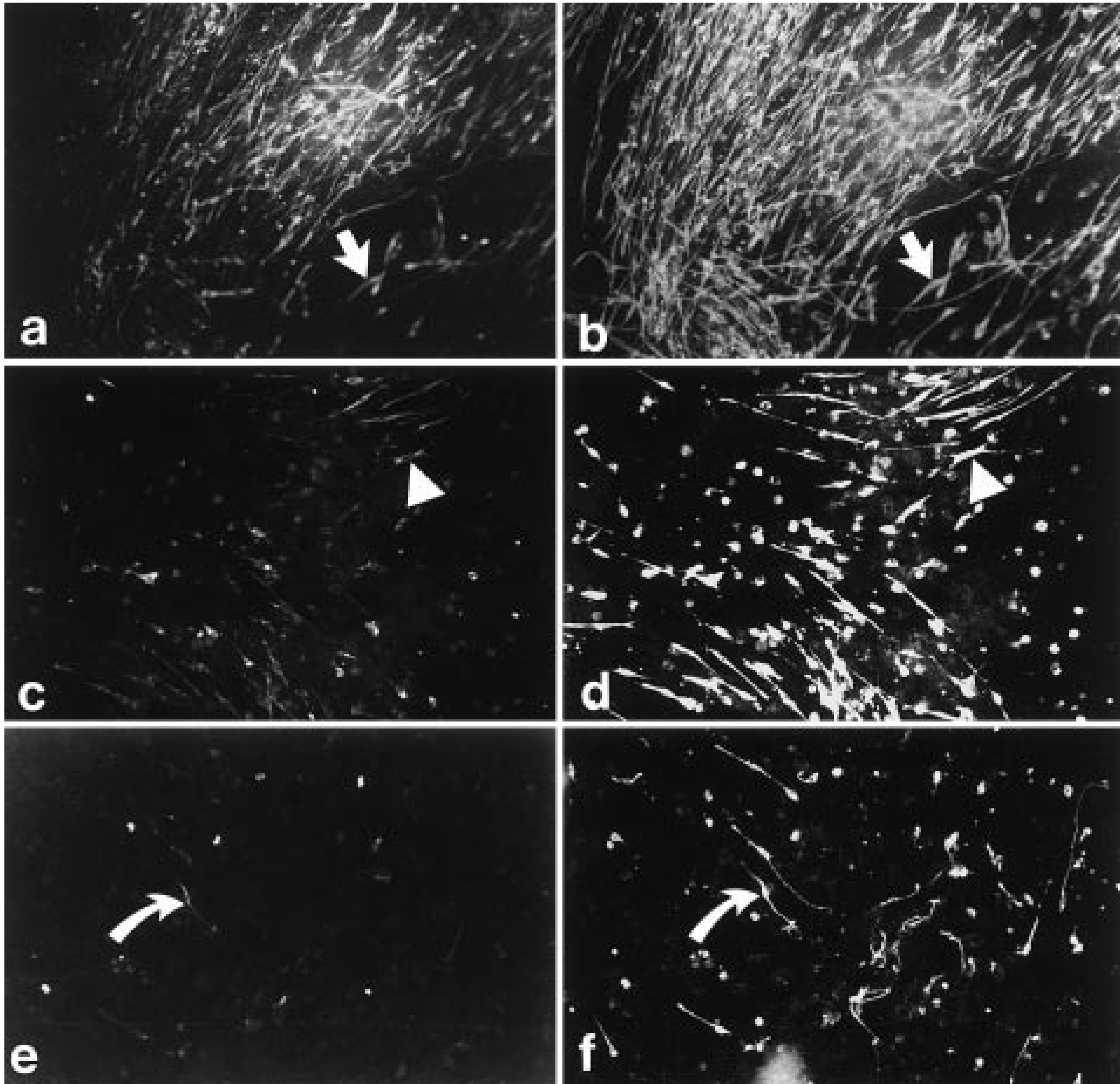

Fig. 4. Muscle cell cultures prepared from 11 day (a and b) and 13 day (c and d) myotome, and 16 day foetal forelimb (e and f) were grown for 3 days *in vitro*. Cell cultures were stained with antibodies 96J to slow (a, c, e), and F2 to fast myosin heavy chains (b, d, f) by double immunofluorescence procedures. Similar arrows indicate the same areas in double-stained cultures. Magnification × 124.

from later foetal rat limb muscles. The reduction in the number of slow MHC-expressing cells during early development is in good agreement with avian *in vitro* studies of myosin heavy chain distribution (Stockdale & Miller, 1987). Vivarelli and coworkers (1988) have also reported the reduction in slow MHC-expressing myotubes in murine muscle cultures prepared from different foetal stages of development, although slow and fast MHCs are present in most myotubes in murine cultures from early foetal

Fig. 3. Serial cross-sections of tibialis anterior muscle from 20 day rat foetus (a–c) and muscle cell cultures prepared from 14 day foetal hindlimb (d and e) and somitic myotome (f and g), grown for 3.5 days *in vitro,* are stained by different myosin heavy chain antibodies by immunoperoxidase procedures. Section (a) is stained with antibody M14 to a slow MHC. Section (b) and muscle cell cultures in (d) and (f) are stained with antibody 96J to slow MHC. Section (c) and muscle cell cultures in (e) and (g) are stained with antibody F2 to fast MHC. Magnifications are ×142 (a–c) and ×161 (d–h).

muscles. The increase in fast muscle fibre type myotubes seen *in vitro* with increasing foetal stage of muscle tissues probably reflects the increased proportion of the fast fibre population during later *in vivo* development of limb muscles compared with the earlier foetal stages of development. Most of this increase in fast fibre type population results from the increase in secondary myotubes, although 'slow' or ventricular myosin is also suppressed in a proportion of the primary generation myotubes during this period (Dhoot, 1986). The number of slow and fast muscle type satellite cells in postnatal skeletal muscles is likewise related to the proportions of the slow and fast muscle fibre types (Rosenblatt *et al*., 1996). This leads to a marked reduction in the number of slow myotubes seen *in vitro* when muscle cell cultures are prepared from most postnatal animal skeletal muscles. Consequently, slow myotubes are observed in cell cultures of only slow-fibre rich muscles such as anterior latissimus dorsi in avian species (Matsuda *et al*., 1983; Feldman & Stockdale, 1991) or, for example, soleus muscle in the rat (Dusterhoft & Pette, 1993; Rosenblatt *et al*., 1996).

As was the case for the chicken and rat foetal skeletal muscles, the presence of distinct slow and fast myotubes was also detected in muscle cell cultures prepared from human foetal skeletal muscles (Ghosh & Dhoot, 1998, and this study). These observations differ from studies of Cho and colleagues (1993) and Edom and colleagues (1994) in which they observed the presence of both slow and fast myosin heavy chains in most *in vitro* myotubes grown from both foetal and postnatal human skeletal muscles. The reason for this difference is not known, but may be related to different culture conditions or the use of different antibodies. The widespread distribution of slow MHC *in vitro* myotubes of human skeletal muscles at most developmental stages may be related to a much higher proportion of slow muscle fibres in most human skeletal muscles compared with most late foetal and postnatal rodent or avian skeletal muscles.

In both mammalian and avian species, only antibodies to slow MHCs identify slow and fast myotube types *in vitro* and in embryonic *in vivo* muscles. Antibodies to FMHCs stain all myotubes equally dark because all *in vitro* myotubes, including those that express slow myosin heavy chains, express fast muscle-like foetal isoforms recognized by the FMHC antibodies used in this study. This is similar to early *in vivo* developing muscles in which the gradually decreasing levels of fast muscle-like foetal isoforms with continued growth becomes apparent in the slow MHC-positive myotubes. In the rat, fast muscle-like foetal and neonatal MHCs are usually not detected in slow muscle fibres after three weeks of postnatal growth (Whalen *et al*., 1981).

The identification of distinct myotube types during early embryonic *in vivo* and *in vitro* development indicates the intrinsic programming of the distinct myotube types before the extrinsic influences start to play a major role. However, not only the *in vitro* studies but also the time at which distinct slow and fast myotubes can be first detected in embryonic muscles of different species has been controversial. For example, while fast and slow myotubes can be identified early in avian embryonic muscles (Crow & Stockdale, 1986; Stockdale, 1992; Williams *et al*., 1992), all myotubes in early rat foetal skeletal muscles have been reported to stain for both fast and slow MHCs using most conventional antibodies (Dhoot, 1986; Narusawa *et al*., 1987; Harris *et al*., 1989; Condon *et al*., 1990). Our studies using a different antibody with a more restricted slow myosin specificity, however, detected the presence of distinct slow and fast myotubes in early foetal rat limb muscles as well as in somitic myotome (Dhoot, 1985, 1986, 1994a; Dhoot & d'Albis, 1993). Such an early identification of mammalian *in vivo* fast and slow myotubes is in good agreement with similar observations of avian embryonic muscles. We therefore support the hypothesis that both avian and mammalian myoblasts are intrinsically heterogeneous, the further development of which is no doubt modulated through extrinsic factors during subsequent growth.

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