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'Early' mammalian myoblasts are resistant to phorbol ester-induced block of differentiation

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Summary

Mesenchymal cells were isolated from somites and limbs of mouse embryos at different developmental stages. When grown in tissue culture, some of the cells underwent muscle differentiation as indicated by synthesis of sarcomeric myosin, acetylcholine receptor and, in the case of limb cells, fusion into multinucleated myotubes.

When the tumour promoter 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) was added to these cultures, it caused differential effects, depending upon the age of the embryo from which cells were isolated. In cultures of somites or limb bud from embryos up to 12 days *post coitum*, TPA did not interfere with the appearance of differentiated muscle cells. When TPA was added to cultures from older embryos, it inhibited muscle differentiation with an efficiency which increased with the age of the embryo, reaching about 90 % inhibition at 15 days.

After this period, a new population of myogenic cells appeared in the limb, which were able to differentiate in the presence of TPA and represented the great majority of myoblasts after day 18 of embryonic development.

The simplest interpretation of these data can be based on the existence of three major classes of myogenic cell precursors, which appear sequentially during muscle histogenesis: 'early' myoblasts, which appear resistant to tumour promoters; 'late' myoblasts, whose differentiation is inhibited by tumour promoters and 'satellite' cells which, like early myoblasts, show no sensitivity to TPA.

Key words: myogenic lineage, phorbol esters, muscle differentiation, somites, mouse, TPA.

Introduction

The histogenesis of skeletal muscle fibres lasts from the somitic period to the end of postnatal somatic growth. Skeletal myoblasts, which give rise to muscle fibres, are in fact present in the mesoderm for a comparably long period. Although those cells present around muscle fibres during postnatal life are differentially termed 'satellite' cells (Mauro, 1961), they have long been assumed to be part of an homogeneous population of myogenic cell precursors.

However, after the initial work of Hauschka and colleagues, evidence for myogenic cell heterogeneity has been accumulating at an increasing pace. In addition to the originally reported differences in morphology and growth requirements (Bonner & Hauschka, 1974; White *et al.* 1975), it has been shown that myotubes derived from early myoblasts synthesize both fast and slow myosin heavy chains, whereas those derived from late myoblasts synthesize only fast myosin (Miller *et al.* 1985). Moreover, differences in the proportion of myosin light chains have been reported between early and late myoblasts (Mouly *et al.* 1987). More recently, three subclasses of early myoblasts, capable of generating slow, fast or mixed muscle clones have been identified (Miller & Stockdale, 1986*a*) and it has been proposed that these different myogenic cells contribute to the formation of different classes of primary myotubes (Miller & Stockdale, 1986*b*).

In previous work, we reported the existence of differential sensitivity to phorbol ester tumour promoters between late myoblasts and satellite cells

(Cossu *et al.* 1983, 1985). In this paper, we have extended the analysis of the effect of tumour promoters on 'early' myoblasts and report here that these cells, unlike 'late' myoblasts, are completely resistant to the drug. The possible significance of this result in terms of myogenic cell-lineages is discussed.

Materials and methods

Cell cultures

Cells were cultured from somites and limbs of mouse embryos of different ages (days *post coitum*) as described before (Vivarelli & Cossu, 1986; Pacifici *et al.* 1980). Satellite cells were cultured as described (Cossu *et al.* 1983). Briefly, the somites or the limbs were isolated from the embryo and, after removal of the skin (from 14-day or older embryos), digested with 0·05 % trypsin (Difco) in phosphate-buffered saline (PBS) for 15 min at 37 °C with occasional shaking. In the case of satellite cells, a predigestion with 0·1 % collagenase (w/v) and 0·1 % hyaluronidase and three sequential digestions with trypsin were required. After the proteolytic digestions, the tissues were fragmented by repeated pipetting, the debris was removed by filtration through a sterile nylon gauge and the cells were collected by centrifugation. All cultures were grown in Dulbecco's basal medium supplemented with 15 % horse serum and 5 % embryo extract. TPA (10^{-7} M final concentration) was diluted from a ×1000 solution in ethanol into complete medium and was added daily to the cultures. To avoid growth stimulatory effects of medium changes, the drug was added to conditioned medium from sister cultures.

Immunocytochemistry

At the indicated periods of culture, cells were fixed with 50 % ethanol–50 % acetone at −20 °C for 10 min, washed in PBS and incubated with the anti-myosin monoclonal antibody MF20 (undiluted supernatant from growing hybridoma for 30 min at 25 °C) and then with a goat anti-mouse Ig conjugated with rhodamine from Cappel (at 1:30 dilution for 30 min at 25 °C) according to a procedure described elsewhere (Cossu *et al.* 1985). The MF20 antibody (Bader *et al.* 1982) recognizes all sarcomeric myosins but does not react with nonmuscle myosins. In the present paper, we assumed as a criterion for cell differentiation the fluorescence staining with anti-myosin antibody.

Assay for acetylcholine receptors (AChR)

The number of specific ^{125}I-α-bungarotoxin (BTX) (Amersham, specific activity 230 Ci mmole^{-1})-binding sites on the surface of myotubes was measured in triplicate cultures (Cossu *et al.* 1982) by incubating cells with 10 nM-^{125}I-α-BTX in Minimum Essential Medium (MEM) supplemented with 1 % horse serum; nonspecific binding was measured in sister wells preincubated for 1 h with 150 μM-d-tubocurarine (d-TC, Calbiochem) and was subtracted from the values obtained.

Results

4 h after plating, TPA was added to cultures from somites and limb buds of 10-day mouse embryos and from limbs of 12-, 14-, 16- and 18-day embryos. After 5 days *in vitro*, both control and TPA-treated cultures were assayed for the presence of myosin-positive cells by staining with a specific antibody. Parallel cultures were incubated with iodinated α-bungarotoxin and the amount of acetylcholine receptor expressed by these cultures was measured in both control and TPA-treated cells.

Fig. 1 shows the presence of myosin-positive cells in both control and TPA-treated cultures, established from limbs of mouse embryos of different ages. In the case of cultures from somites of 10-day embryos, the same number of myosin-positive cells could be observed in both control and treated cultures. Examples of these cells, which are mostly mononucleated (Vivarelli & Cossu, 1986) are shown in Fig. 1A,B. A similar result was obtained when cultures were established from limbs of 10- and 12-day embryos. In the case of 14-day embryos, the number of myosin-positive cells was clearly reduced (ranging from 50 to 70 % of control) in TPA-treated cultures (Fig. 1C,D). As reported previously (Cossu *et al.* 1983), in cultures from 16-day embryos, the appearance of myosin-positive cells was inhibited over 90 % by TPA (Fig. 1E,F). In contrast, at later developmental stages, the percentage of cells, that were sensitive to TPA progressively decreased and, at birth, almost all myogenic cells were again TPA-resistant (see Cossu *et al.* 1983).

To evaluate quantitatively this phenomenon, we examined the expression of acetylcholine receptors (AChR) in the same experimental conditions. Fig. 2 shows the appearance of AChR, measured by specific binding of iodinated α-bungarotoxin, at different days *in vitro* in both control and TPA-treated cultures, established from 11- (Fig. 2A) or 16- (Fig 2B) day-old embryos. The figure shows that early myogenic cells accumulate AChR whether or not TPA is present in the culture medium. In contrast, late myoblasts do not accumulate AChRs when treated with TPA.

By expressing the amount of BTX bound by TPA-treated cultures as % of their own controls, a developmental pattern of TPA resistance of myogenic cells can be obtained (Fig. 3). This pattern shows a

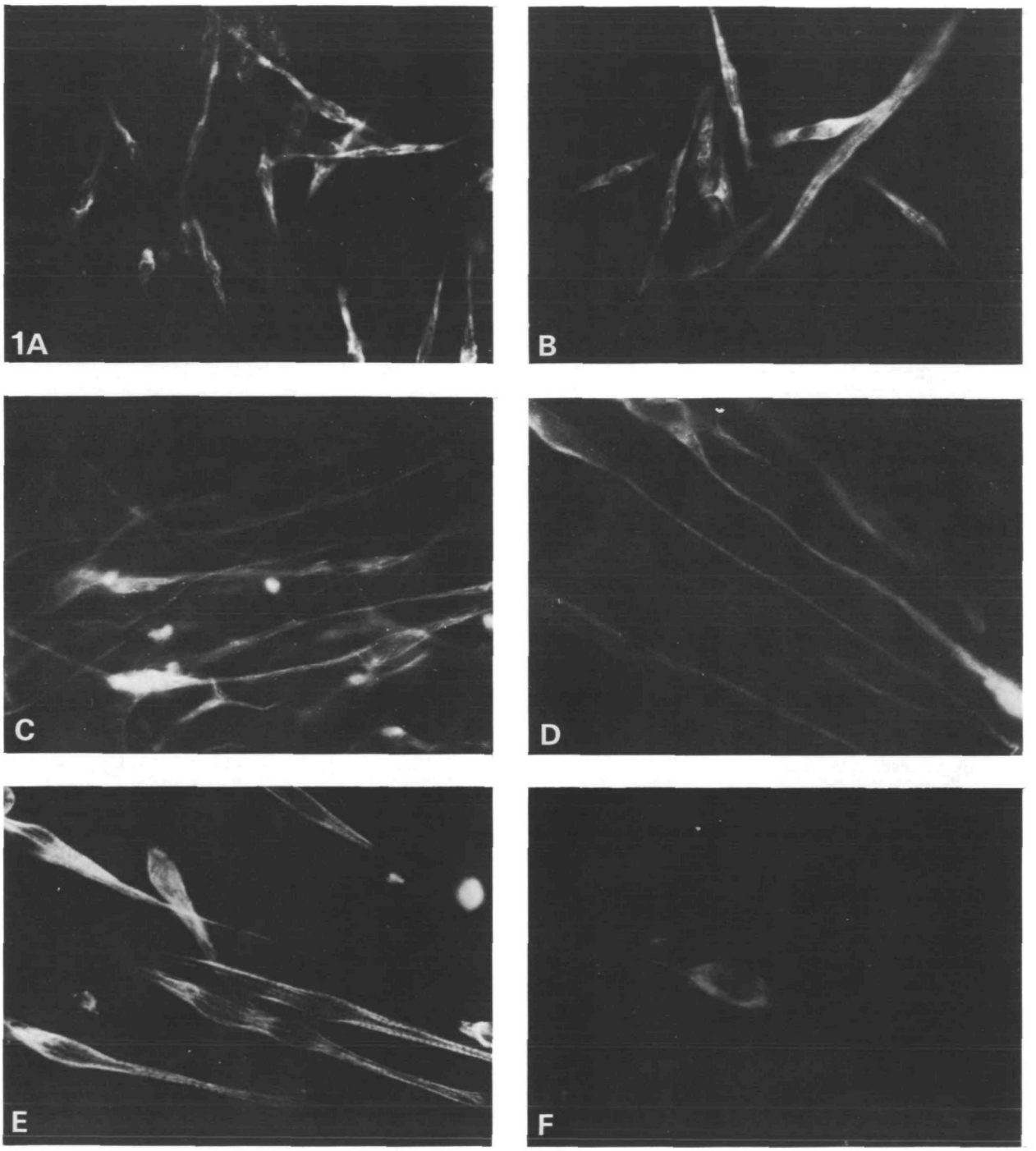

Fig. 1. Immunofluorescent pictures of Control (A,C,E) and TPA-treated (B,D,F) cultures of myogenic cells isolated from 10-day somites (A,B), 14-day limb buds (C,D) and 16-day limbs (E,F), stained with MF20 after 5 days *in vitro*. Note well-developed cross striations in the thin muscle cells (D) as well as in multinucleated myotubes (E); a single TPA-resistant, differentiated cell is shown in F. ×150.

biphasic curve: while at early stages most of the cells appear resistant to phorbol esters, during the following days of development, the population of myogenic cells becomes progressively more susceptible to the TPA-induced block of differentiation, to reach an almost total inhibition at day 16 of development. After this day, however, the cells become again progressively more resistant to TPA and, around the final days of embryonic development, they become totally insensitive to the drug. Data presented in the second part of Fig. 3 have already been published (Cossu *et al.* 1983) but are included here to show the

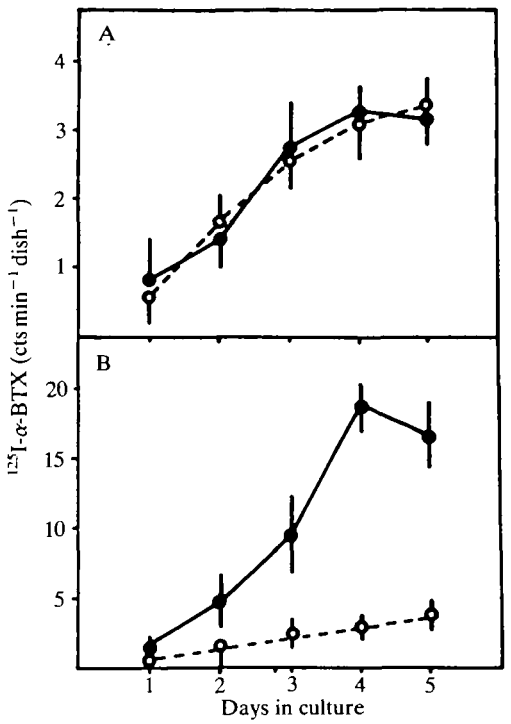

Fig. 2. Levels of ACh receptors in cultures of embryonic muscle cells. Cultures were established from limbs of 11- (A) or 16- (B) day-old mouse embryos and grown in the absence (●——●) or in the presence (○- - -○) of 10^{-7} M-TPA. ACh receptors were measured from day 1 to day 5 of culture by binding with ^{125}I-α-bungarotoxin (Cossu *et al.* 1983). Each point is the mean ± standard deviation of three separate experiments (each performed in triplicate).

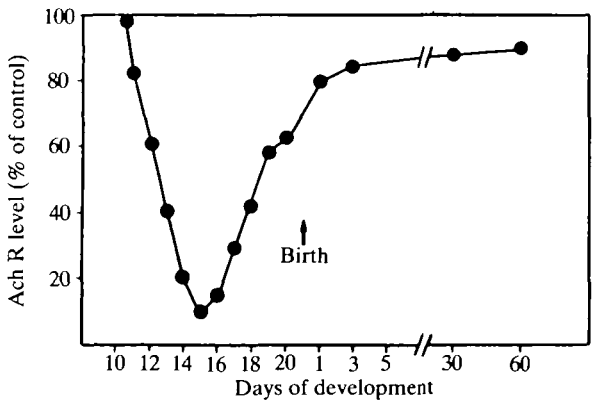

Fig. 3. Developmental pattern of TPA resistance in myogenic cells. Cultures were established from mouse embryos of different ages and from newborn and adult mice. Cultures were grown in the absence and in the presence of 10^{-7} M-TPA. ACh receptors were measured in 5-day-old cultures by binding with ^{125}I-α-bungarotoxin (Cossu *et al.* 1983). The levels of ACh receptors in TPA-treated cultures were expressed as a percentage of the levels of the corresponding control cultures.

complete developmental pattern of TPA-sensitivity of murine myogenic cells.

Discussion

The data reported in this paper show that phorbol ester tumour promoters show a differential effect on myogenic cells, depending upon the age of the animal from which the cells were isolated. Previously, we reported that satellite cells from mammalian muscle are capable of differentiating in the presence of phorbol esters at concentrations which completely block differentiation of myoblasts (Cossu *et al.* 1983, 1985). These myoblasts, however, are usually isolated from embryos at an advanced stage of development, i.e. 11 days in the chick, 15–17 days in rodents (Cohen *et al.* 1977; Cossu *et al.* 1983). Recently, several biochemical and immunocytochemical (Miller *et al.* 1985; Miller & Stockdale, 1986*a*,*b*; Mouly *et al.* 1987) data have confirmed and extended the original observations of differences in morphology and growth requirements (Bonner & Hauschka, 1974; White *et al.* 1975) between myoblasts from post-somitic stages and myoblasts from the embryonic periods described above. Based on these data, it is now possible to divide embryonic myoblasts into two main classes: 'early' (which can be further subdivided into 'fast', 'slow' and 'mixed') and 'late'. It is possible that these different classes contribute to different phases of muscle fibre histogenesis. Therefore, it is important to identify biochemical or immunological markers that distinguish between these cell types and, possibly, between the fibres which they generate. In this context, we now report that early myoblasts differ from late ones because they are completely resistant to TPA.

It should be noted that the total extent of muscle differentiation is much lower in cultures of early myoblasts (Bonner & Hauschka, 1974; White *et al.* 1975; Miller *et al.* 1985; Cossu *et al.* 1985; Mouly *et al.* 1987): yet, the expression of the markers examined was clearly induced throughout the culture in both control and TPA-treated cells at an identical rate, whereas any inhibition larger than 10% would have been clearly detected.

The percentage of cells resistant to TPA *in vitro* can be used to monitor the disappearance of early myoblasts during mouse embryogenesis. The pattern obtained matches very well with the disappearance of early myoblasts in chick (White *et al.* 1975) and also, with the disappearance of myogenic clones (in mouse) that require conditioned medium to differentiate *in vitro* (Cossu, unpublished data).

As to the biochemical basis of this TPA resistance, which hopefully might shed light on basic biochemical differences among different myogenic cells, we have

recently reported that the resistance to TPA of satellite cells is probably dependent on the phospholipid composition of their membranes, since it is possible to restore TPA sensitivity by liposome-delivered phosphatidylserine (Cossu *et al.* 1986). Unfortunately, 'early' myoblasts are extremely sensitive to this treatment and therefore we could not rule out the possibility that, in this experiment, differentiation was blocked simply because myogenic cells were killed. In any case, we may conclude that TPA resistance is common to 'early' myoblasts and to satellite cells. Whether this implies any possible genealogical relationship between these two cell types cannot be determined on the basis of the present evidence, but remains, in our opinion, a possibility for future investigations.

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