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Embryonic development of *Xenopus* studied in a cell culture system with tissue-specific monoclonal antibodies

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Summary

An *in vitro* microculture system of early gastrula cells of *Xenopus laevis* has been developed; deep layer cells from the lateral marginal zone (prospective somite region) or ventral ectoderm (prospective epidermis region) were fully dissociated, and the desired number of each (1–100) was distributed into a microculture well and cultured under appropriate conditions. When examined with the tissue-specific Mabs (Mu1 for muscle and E3 for epidermis, respectively), a substantial portion of the deep layer cells from the two regions followed their respective normal embryonic fates. It was found that reproducible cellular differentiation was dependent on the intimate reaggregation of dissociated cells and on the size of the resultant aggregate. About 20 lateral marginal zone cells were found to be sufficient, when put into a culture well, for supporting successful muscle differentiation, whereas about 100 ventral ectoderm cells were necessary for epidermal differentiation.

Key words: *Xenopus* embryo, monoclonal antibody, immunofluorescence, microculture, dissociated cells.

Introduction

The aim of the present study was to develop an *in vitro* microculture system with the aid of tissue-specific Mabs for examining the differentiation of early gastrula cells of *Xenopus laevis*. Care has been taken to obtain reproducible states of differentiation in culture, because it has been thought extremely difficult in this species to direct the marginal zone cells of early gastrula to differentiate in *in vitro* cultures on a small scale (Jones & Elsdale, 1963). Furthermore, we tried to minimize the number of cells that were initially added to the wells, while still obtaining reproducible differentiation. This is to facilitate immunofluorescent examination and also to increase the total number of culture wells that can be set up when the quantity of experimental material from a single experiment is limited. With these approaches, the differentiation of early gastrula cells was analysed under a variety of experimental conditions and compared in a quantitative manner in each experimental series. It was demonstrated that, even in typical cases of the autonomous differentiation such as had been seen in epidermal or muscular development (Slack, 1983), interactions among the homologous cells of a prospective region played a significant role.

Materials and methods

Animal care, generation of hybridomas and immunohistochemistry on frozen sections

These were done in essentially the same way as described in the previous paper (Mitani & Okamoto, 1988).

In vitro *microculture system for early gastrula cells*

Early gastrula embryos (stage $10\frac{1}{4}$–$10\frac{1}{2}$ according to Nieuwkoop & Faber, 1967) were dejellied in 2% cysteine hydrochloride (pH 7·9) and kept in a modified Barth solution (MBS; Gurdon, 1977) on ice until the subsequent operation step. All solutions subsequently used contained 1% bovine serum albumin (BSA, Fraction V, Wako) and 20 $\mu g\,ml^{-1}$ gentamycin. About 20 to 40 embryos were manually removed from the vitelline membrane and dissected in MBS. The removed fragments were disaggregated by incubating in Ca^{2+}-, Mg^{2+}-deficient MBS (pH 7·2) for about 30 min on a 1% agarose cushion. During this period, the outer pigmented layer of each fragment was removed manually and discarded. After cells had been thoroughly dissociated, collected by a hand centrifuge and resuspended in standard MBS, the desired number of cells (about 1–100 in 10–20 μl) were distributed into microculture wells (total about 200–400) of Terasaki plates with a micropipette. All the wells had been prewashed with MBS containing 1% BSA overnight. The purpose of preparing a large pooled suspension of dissociated cells prior to distribution was to circumvent the influence of possible variations in individual embryos and operations (staging, position of fragments, extent of damage to cells etc).

The plates were then, unless otherwise noted, centrifuged at a low speed in an angled position to collect cells at the bottom rim of each microculture well under nearly the same conditions. This procedure encouraged reaggregation of the cells, which favoured the differentiation of the dissociated early gastrula cells in culture (Jones & Elsdale, 1963, see also text). After overflowed medium had been aspirated from the plates and 20 μl of fresh MBS (pH 7·8) was added to each well, the plates were centrifuged again. This procedure was repeated twice more. After the final aspiration, each culture well contained 12 μl of culture medium. The plates were put into chambers that were moisturized with MBS (BSA free), with or without further addition of 12 μl MBS to each well, and kept at 22·5 °C until fixation. For cultures longer than 24 h, half the culture medium was substituted by 66 % RPMI 1640 containing 10 mM-$NaHCO_3$ and 1 % BSA after around 20 h of culture.

Staining of cultured cells

Cultured cells were fixed with 0·5 % paraformaldehyde in MBS containing 0·1 mg ml^{-1} poly-L-lysine for 16 h at 0 °C and washed overnight in PBS containing 50 mM-glycine in a refrigerator. Poly-L-lysine was found to facilitate attachment of cultured cells onto wells.

For indirect immunofluorescence, each well received 10 μl of a first layer Mab containing 1 % (for E3) or 0·25 % (for Mu1) NP-40. After incubation for 2 h at room temperature, the plates were washed by gently dipping into PBS. The washing procedure was repeated several times over 3–4 h. The plates were finally removed from PBS and excess PBS was aspirated. Each well then received 10 μl of FITC-conjugated second layer antibody containing 0·25 % NP-40 and incubated 1 h at room temperature. Washings were repeated as before. Nuclear staining was carried out with 0·5 μg ml^{-1} of DAPI in order to visualize the overall cell arrangement. The plates were mounted on epiluminescent fluoroscope in inverted position, observed and photographed.

Results

Specificities of Mabs in situ

Typical binding patterns of two Mabs (E3 and Mu1) to larval sections (stage 37/38) that were used in the subsequent microculture studies are presented in Fig. 1. The larval epidermis in *Xenopus laevis* consists of two (outer and inner) layers, each mainly derived from the outer heavily pigmented layer and inner less pigmented layer of gastrula ectoderm, respectively. As seen in Fig. 1B, E3 binds to both layers. With higher magnification, the E3 antigen is seen mainly in the peripheral region within the cells of both layers. The inward extension, though small, of the antigen distribution and resistance to detergent (data not shown) suggest that the E3 antigen molecule does not lie in plasma membrane itself but is associated with some peripheral cytoskeletal structure. The E3 antigen was first detected at stage 21 and stage 29/30 in the outer and inner layers of epidermis, respectively.

The spatial specificity of the Mab Mu1 was found to be high. It binds to muscle cells in the myotome (Fig. 1C) but not to heart muscle or intestinal smooth muscle cells (data not shown). With higher magnification, the Mu1 antigen was seen to be distributed throughout the cytoplasm, except the nucleus, though not uniformly. The Mu1 antigen was first detected at stage 21.

Muscle and epidermal cell differentiation in the microculture system

Deep layer regions of the lateral marginal zone (about 45° wide at dorsal side, prospective somite region; Keller (1976)) of early gastrula (stage $10\frac{1}{4}$–$10\frac{1}{2}$) were processed as described in Materials and Methods. Immediately following the centrifugation step, the dissociated cells began to attach to each other and, after 1 to 2 h in culture, the outline of individual cells became unresolved under a phase-contrast microscope. The resultant ball of tightly coherent cells spreads into thin sheets of cohesive cells by 38 h in culture (Fig. 2A*a1*,*a2*). We have monitored the differentiation

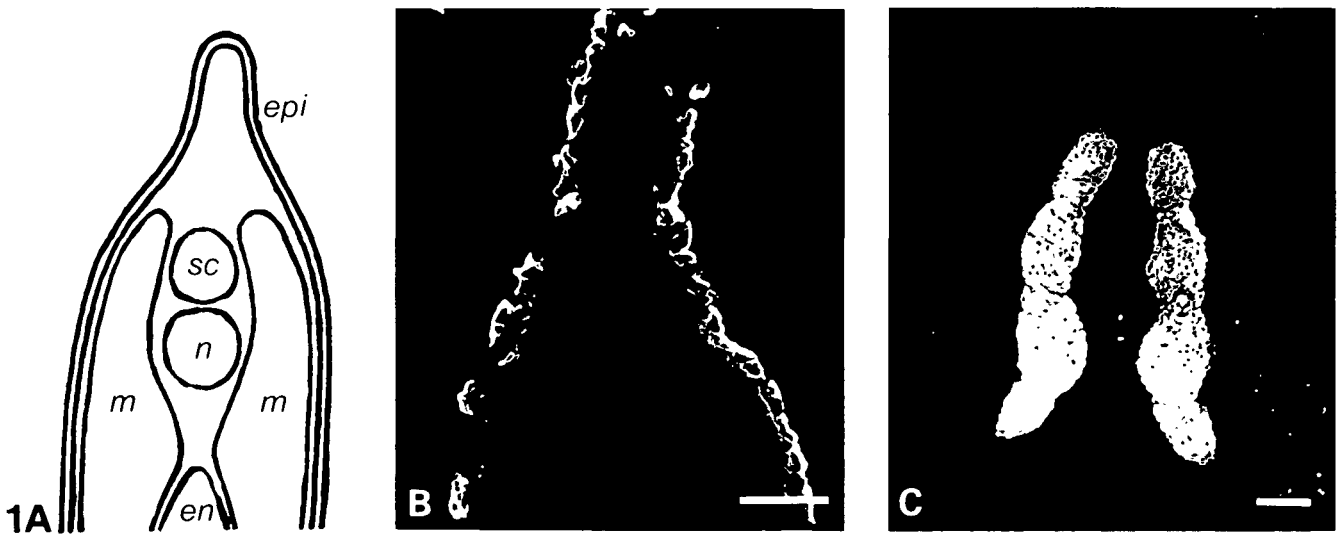

Fig. 1. Tissue specificities of monoclonal antibodies, E3 and Mu1. (A) Schematic drawing of gross anatomy in larval cross-section used (stage 37/38). (B) Binding of E3 to epidermis. (C) Binding of Mu1 to myotome muscle. Abbreviations: *epi*, epidermis; *sc*, spinal cord; *n*, notochord; *m*, myotome; *en*, endodermal mass. Bars = 100 μm.

of muscle cells in these cultures with Mab Mu1 (Fig. 2A*a3*). There was no sign of the epidermal differentiation, as judged by the binding of Mab E3 (data not shown).

When the deep layer cells from the ventral ectoderm of early gastrula (stage 10¼), which is the major source of embryonic epidermis (Keller, 1976), were cultured under the same condition as described for those from the lateral marginal zone, their behaviour was very similar, at least for the first few hours in culture. The ball of the ectodermal cells, however, did not flatten or spread in the subsequent culture period in most instances. Instead, they began to swim around in the culture well by 30 h in culture. With a rather vigorous

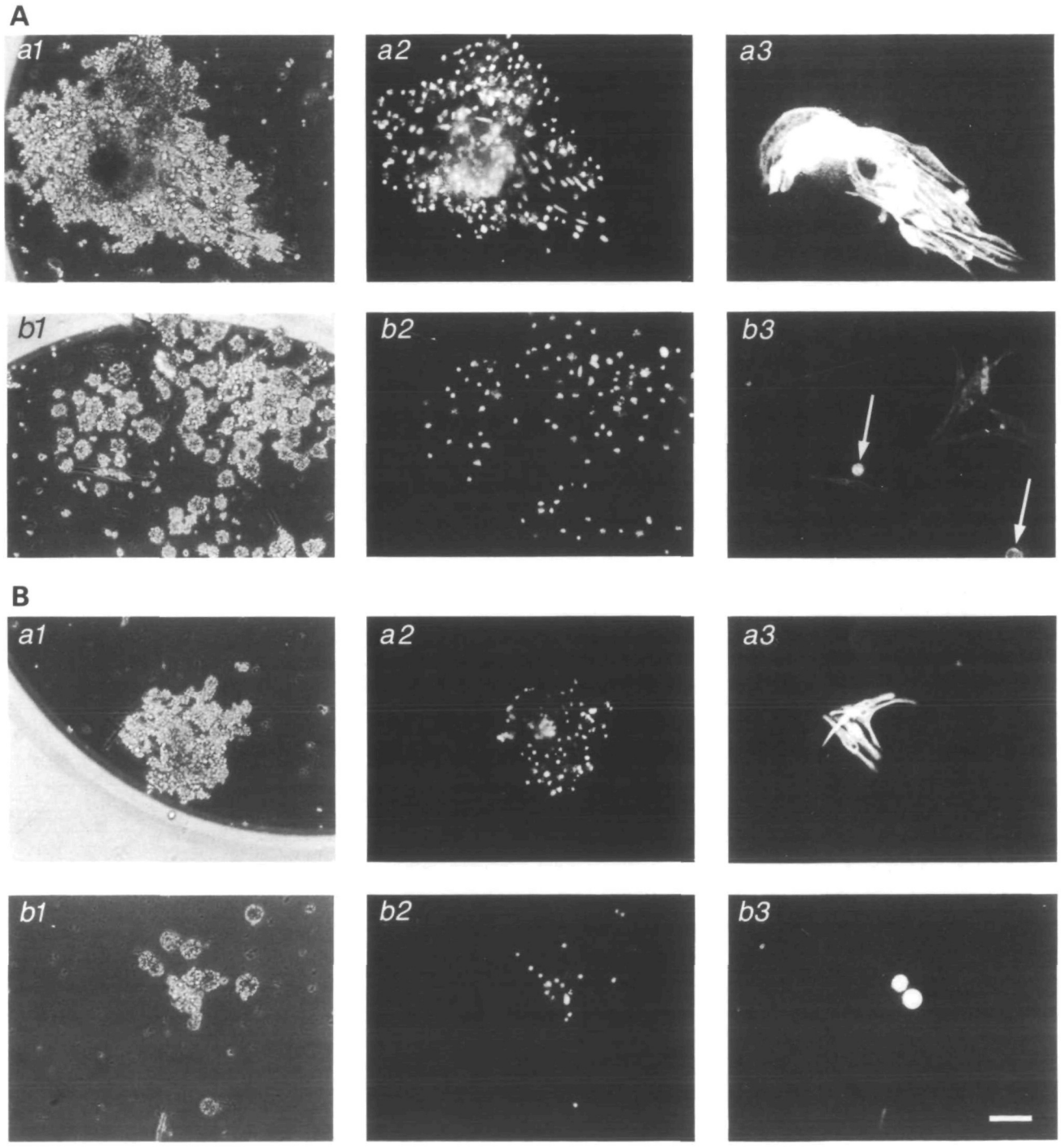

Fig. 2. Development of deep layer cells from the lateral marginal zone of early gastrula in microculture wells. A suspension of dissociated deep layer cells was prepared from the lateral marginal zone from which about 100 cells (A) or 12 cells (B) were initially added into culture wells; each referred to as 100-cell or 12-cell culture etc., in the text and following figure legends. These cultures were started with (*a1–a3*) or without (*b1–b3*) the centrifugation step for reaggregation. Volume of culture medium was 24 μl for each well. Cultures were fixed after incubation of 36 h and examined for the presence of Mu1 antigen. (*a1*, *b1*); phase-contrast appearance. (*a2*, *b2*); nuclear staining with DAPI. (*a3*, *b3*); Mu1 labelling. Bar = 100 μm.

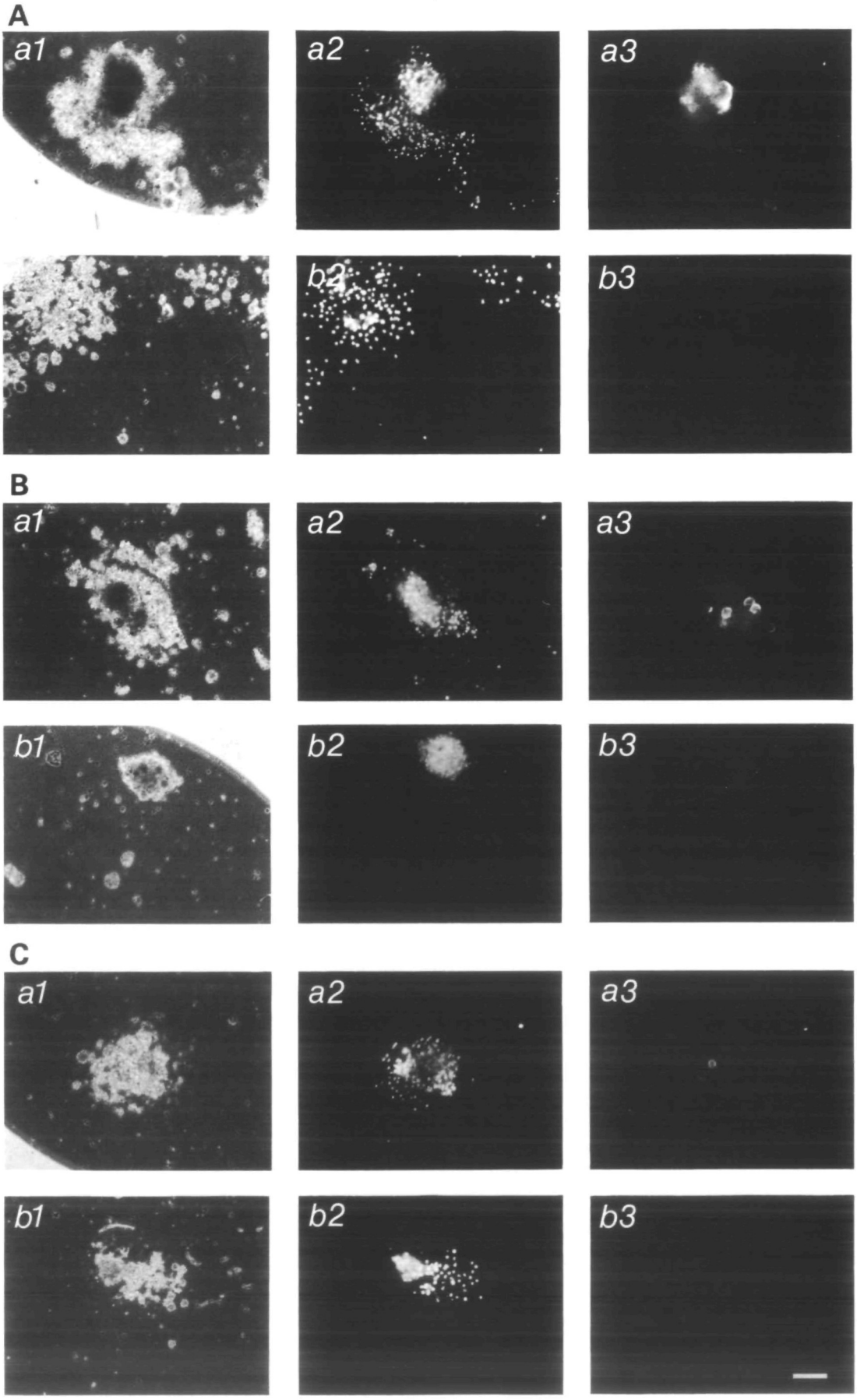
A
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Fig. 3. Development of deep-layer cells from the ventral ectoderm of early gastrula in microculture wells. In A, 100-cell cultures were started with (*a1–a3*) or without (*b1–b3*) the centrifugation step. In B and C, 50-cell cultures (B) or 25-cell cultures (C) were started with the centrifugation. After 36 h of incubation, cultures were fixed and examined for the presence of E3 antigen. Typical examples of positive (*a1–a3* in B and C) and negative (*b1–b3* in B and C) wells for E3 antigen are shown. Volume of culture medium was 24 μl for each well in A to C. (*a1*, *b1*); phase-contrast appearance. (*a2*, *b2*); nuclear staining. (*a3*, *b3*); E3 labelling. Bar = 100 μm.

motion, most of the swimming balls partly ruptured and some cells inside them were scattered over the bottom of culture well (Fig. 3A*a1*,*a2*). We have detected an epidermal antigen in the surface cells of these cultured balls that binds Mab E3 (Fig. 3A*a3*). Muscular antigens could not be detected in these ectodermal cell cultures as judged by the lack of binding of Mu1 (data not shown).

Both Mu1 and E3 antigens are expressed *in vitro* at the appropriate time when compared to the timing of their expression in intact embryos (data not shown). Combined with the data on the absence of aberrant differentiation, we may conclude that our microculture system of early gastrula cells reflects their *in situ* situation to a considerable extent.

Effects of aggregated versus *dispersed culture condition and the size of aggregate on epidermal and muscle differentiation; assessment of the role of cell interaction*

This part of the present study was undertaken to investigate the effects of varying the conditions of culture on the differentiation of both muscle and epidermal cells from early gastrula. The results are shown in Figs 2 and 3 (some examples of typical staining pattern with Mu1 and E3, respectively) and in Fig. 4 (cumulative data). Our major conclusion is that reproducible cell differentiation is dependent on the intimate reaggregation of dissociated cells and the size of resultant reaggregate. This is determined by the number of cell initially added to culture wells.

The effect of the initial centrifugation step, used to facilitate reaggregation, is profound in cultures of ectoderm cells over the range of 12- to 100-cell culture (see Fig. 2 legend), as judged by E3 binding (solid line *versus* broken one in Fig. 4A, each with or without the centrifugation step). Typical examples of the two types of culture of 100 cells are compared in Fig. 3A. In cultures of lower cell number, epidermal differentiation, if any, is extremely poor even with the centrifugation step, as shown in Fig. 3B and C, in which E3 negative wells are also included for comparison. There are just a few cells seen expressing E3 antigen.

The extent to which muscle cell differentiation from the dissociated lateral marginal zone cells depends on reaggregation is much smaller than the epidermal cell differentiation from the ventral ectoderm cells, as assessed by Mu1 binding (solid lines *versus* broken one

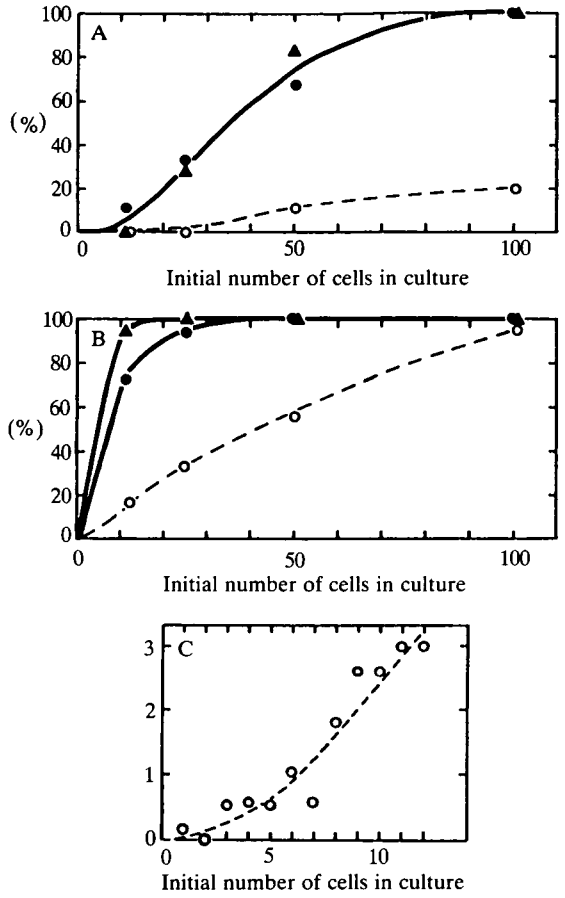

Fig. 4. Dependence of epidermal (A) and muscular (B,C) cell differentiation on reaggregation and the initial number of cells in the culture well. About 10 to 100 deep layer cells from the ventral ectoderm (A) or lateral marginal zone (B) of early gastrula were initially added to culture wells. These cultures were started with (▲, ● in A and B) or without (○ in A and B) the centrifugation step for reaggregation. Volume of culture medium was adjusted to 12 μl per well (▲ in A and B) or 24 μl per well (●, ○ in A and B). In C, initial number of marginal zone cells in each well (1–12) were directly counted under microscope just after centrifugation. After 36 h of incubation, cultures were fixed and examined for the presence of E3 (A) or Mu1 (B,C) antigen. In A and B, the proportion of positive wells for the respective antigens is plotted against initial number of cells in well. The total number of culture wells examined was 16 to 18 for each point. In C, mean number of Mu1-positive cells per well is plotted against initial number of cells in well.

in Fig. 4B). In nearly all the cultures of 100 cells, we could detect Mu1-positive muscle cells, irrespective of the centrifugation step. The extent of differentiation is, however, considerably different between the two types of culture. Both the number of differentiated muscle cells and the amount of Mu1 antigen in individual cells appear to be larger in cultures started with the centrifugation step than those in cultures without it (Fig. 2A). Furthermore, in the dispersed culture system some peculiar cells are occasionally encountered that are round in shape, yet nevertheless express Mu1 antigen

(Fig. 2A*b3*, arrows). These cells might also be muscle in nature, but fail to extend over the substratum of the culture well. The difference between the two culture systems is more clearly shown in 12-cell cultures (Fig. 2B), where individual Mu1-positive cells can be easily identified and counted (Fig. 2B*a3* and *b3*). While only six round Mu1-positive cells were scored in the 18 dispersed culture wells examined, thirty-eight extended cells in addition to nine round Mu1-positive cells were scored in the 18 centrifuged culture wells.

When fewer (1–12) cells were placed in wells and the differentiated Mu1-positive cells were counted, a non-linear relationship was obtained (Fig. 4C); larger initial cell number yielded higher frequency of the muscle cell differentiation. In summary, the reaggregation of the larger number of cells appears to facilitate the differentiation of marginal zone cells into muscle cells. It was noteworthy, however, that even a single cell was capable of differentiating into muscle cell in some cases (6 out of 45 single cells in Fig. 4C and 1 out of 109 in the other series of experiments).

Discussion

Isolates from the lateral marginal zone and the ventral ectoderm in *Xenopus* early gastrula follow their normal embryonic fates in explant culture; autonomous differentiation (Slack, 1983). We have shown in the present study that this autonomous type of differentiation is dependent on the intimate reaggregation of homologous cells in culture. From Fig. 4A and B, we could estimate in the aggregated culture that a minimum of about 20 cells of the lateral marginal zone and 100 cells of the ventral ectoderm are required for reproducible muscle and epidermal differentiation, respectively. These figures suggest that dependence on cell interaction is much greater in the development of ectoderm cells than for the marginal zone cells (both inner layer cells). Alternatively, it is possible that the critical parameter was not the cell number but the cell mass, because animal pole cells were expected to be considerably smaller than marginal zone cells. This seems, however, not to be the case for the experiment shown in Fig. 4, in which the ectoderm cells were prepared from a rather broad ventral area of stage $10\frac{1}{4}$ embryos, while the marginal zone cells were from embryos at a stage about 30 min more advanced. Mean diameter of the ventral ectoderm and lateral marginal zone cells in the above preparations was $32{\cdot}8 \pm 4{\cdot}0\,\mu m$ ($n = 64$) and $34{\cdot}8 \pm 6{\cdot}4\,\mu m$ ($n = 67$) (mean ± S.D.), respectively, which would give the cell mass ratio of 1:1·2. On the other hand, we could not exclude the possibility at present that the 30 min difference in the developmental ages would affect the results.

Our observation on aggregation dependence of muscle differentiation is consistent with those in the previous studies (Gurdon *et al.* 1984; Sargent *et al.* 1986), in which the transcription of actin genes was completely suppressed in cells that were in a fully dissociated state at the normal time of gene activation (stage 13 onwards). These authors cultured the dissociated cells in Ca^{2+}-, Mg^{2+}-free medium, whereas we cultured them in standard MBS. Thus, we have provided evidence that the suppression of muscle differentiation is not due to the deprivation of the divalent cations, but to a blockade of cell contact.

The aggregation dependence of epidermis differentiation observed in this study, on the other hand, appears inconsistent with results previously reported (Jones & Woodland, 1986; Sargent *et al.* 1986). They showed that from stage 7 onwards in dispersed cultures of embryonic cells no cell interaction is necessary for epidermal differentiation to occur. The molecular probe used in Jones *et al.* (1986) is a Mab (2F7C7) that binds exclusively to the outer layer cells of the embryonic epidermis. Their observation is therefore based mainly on the development of the outer layer cells of ectoderm. In contrast, we have observed the differentiation of epidermal cells from the inner (deep) layer cells of the ventral ectoderm. The discrepancy between the two observations might be reconciled in this way. The differentiation of the cells of the outer and inner layer epidermis differ considerably as judged by spatio-temporal specificities of a variety of epidermal markers (Smith *et al.* 1985; Slack, 1985; Jones & Woodland, 1986 and our present and unpublished observations). It seems that the differentiation of the outer layer is fairly well in advance of that of the inner layer. In addition, the studies of Jones *et al.* (1986) suggest that the outer layer ectoderm cells become specified to form epidermis, that is, become committed to form epidermis when isolated, earlier than the inner layer cells. A similar explanation might be applied to the apparent conflict of our results with those of Sargent *et al.* (1986), only if their probe (DG81 cytokeratin gene) was expressed in the outer layer.

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