

Stimulation of division in mouse 3T3 cells by coculture with embryonic chick limb tissue

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SUMMARY

Two regions of the chick limb bud — the apical ectodermal ridge and the zone of polarizing activity — have been shown to influence cell division and pattern formation during normal development and following surgical manipulation. In this study, using a simple coculture system, together with autoradiography, we have shown that these morphogenetically active regions of the limb bud can stimulate quiescent 3T3 cells to initiate DNA synthesis to a significantly greater degree than comparable but morphogenetically inactive regions of the limb bud.

INTRODUCTION

Cell division plays an important role during the development of the vertebrate limb. In the chick embryo, for instance, the limb buds grow out from regions where the rate of cell division remains high while that of the surrounding flank region falls (Searls & Janners, 1971). Later in development, limb-bud outgrowth and the formation of the proximodistal axis are dependent on the continuing presence of the apical ectodermal ridge (AER) (Saunders, 1948; Summerbell, Lewis & Wolpert, 1973; Summerbell, 1974), which maintains a high rate of mitosis in the distal mesoderm (Hornbruch & Wolpert, 1970). A group of cells at the base of the limb bud, known as the zone of polarizing activity (ZPA) is considered to be necessary for the formation of the anteroposterior axis (Tickle, Summerbell & Wolpert, 1975; Summerbell, 1979). Grafting an additional ZPA to the anterior margin of another limb bud rapidly induces cell division in the host tissues and results in the formation of supernumerary structures (Saunders & Gasseling, 1968; Cooke & Summerbell, 1980).

Previous *in vitro* experiments have shown that both the AER and the ZPA are required for the survival and growth of chick limb tissue explants (Cairns, 1975; MacCabe & Parker, 1975; MacCabe, Calandra & Parker, 1977; Reiter & Solursh, 1982). In all these studies, however, the responding tissues were too complex to give a clear idea of the cellular processes involved. In an attempt to quantify the growth-promoting effects of the AER and the ZPA we devised a simple assay system, derived from those normally employed for the detection of growth factors. This revealed striking variations in the ability of relatively similar regions of the

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embryonic chick limb to induce DNA synthesis in a population of target cells. We chose the embryonic mouse fibroblast line NIH-3T3 as our responding cells, since these are readily obtainable, they are more homogeneous than primary embryonic cultures and they survive under known low serum conditions.

In the embryo an additional ZPA exerts its effects between 24 and 36 h after grafting (Smith, 1980). Consequently we cocultured our 3T3 cells with the chick tissue for 24 h before adding tritiated thymidine. The response to different numbers of tissue pieces was investigated to clarify the effect of dose. In addition, conditioned media were used to see whether cell-tissue contact was necessary to stimulate a response. A small number of grafting experiments were also carried out on the limb to confirm that distal and anterior tissue did not produce duplications along the anteroposterior axis in the same way as the ZPA.

MATERIALS AND METHODS

All the medium used in this study was alpha-modified Eagle's Medium (Stanners, Eliceiri & Green, 1971) (Flow, Irvine, Scotland) without added nucleosides or deoxynucleosides, with antibiotics (penicillin G, $75 \mu\text{g}.\text{ml}^{-1}$ and streptomycin sulphate, $50 \mu\text{g}.\text{ml}^{-1}$; Glaxo, Greenford, England) and containing 5% or 0.5% (low serum medium) (v/v) heat-inactivated foetal calf serum (Sera-Labs, Crawley Down, England). All cell and tissue culture was carried out at 37°C with an atmosphere of 5% (v/v) CO_2 in air. Cells of the mouse fibroblast line N.I.H. 3T3 (a gift from C. M. Isacke, Salk Inst., Calif.) (Todaro & Green, 1963) were routinely grown in 25 cm^2 tissue culture flasks (Nunc, Paisley, Scotland) before they were transferred to tissue culture chamber slides, well size 0.89 cm^2 (Flow), at a concentration of 10^4 cells in 0.5 ml per well. The cells were allowed to attach and approach confluence before lowering the serum level to 0.5%. The 3T3 cells were then left in low serum medium for at least 24 h, to reduce the numbers of cell divisions occurring, before the addition of any test tissue.

Fertilized chicken eggs from a local farm were incubated at 38°C and windowed on the fourth day of incubation. The embryos were staged according to Hamburger & Hamilton (1951) and only those between the stages of 19 and 22 were used. Test pieces of tissue were removed from the wing buds using electrolytically sharpened tungsten needles and watchmakers forceps and

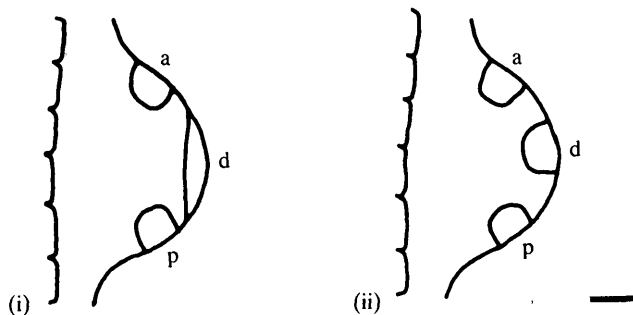


Fig. 1. Diagrams of the chick wing bud at stage 20. (i) Cubes of posterior (p) and anterior (a) tissue of side approximately $200 \mu\text{m}$ and slices of distal (d) tissue approximately $150 \mu\text{m}$ wide were used in the initial experiments. The distal tissue consisted of apical ectoderm plus adjacent mesoderm. The posterior tissue included the region of highest polarizing activity, and both ectoderm and mesoderm. (ii) In the dose-response experiments $200 \mu\text{m}$ sides cubes replaced the slices of distal tissue. Bar = 0.3 mm .

placed in low serum medium until needed. Cubes of side approximately 200 μm were dissected from the region of highest ZPA activity and from the anterior margin. In the initial experiments, slices of distal tissue of about 150 μm wide, including most of the AER, were used but these were subsequently replaced by 200 μm sided cubes in later experiments (Fig. 1). In one experiment the effects of flank tissue were also investigated.

Coculture

Approximately 15 pieces of test tissue were cocultured with the 3T3 cells for 24 h, in low serum medium. At the end of this time the chick tissue was removed and the medium replaced with fresh low serum medium. The fibroblasts were then exposed to tritiated thymidine (methyl- ^3H) thymidine; 40 mCi.mmol $^{-1}$; Amersham International, Amersham, England), at a final concentration of 1 $\mu\text{Ci.ml}^{-1}$ for 18 h. After labelling, the plastic chambers were detached from the slides and the cells prepared for autoradiography. Equal volumes of emulsion (Ilford Nuclear Research, K2 emulsion, Basildon, England) and distilled water containing 2% (v/v) glycerol were mixed at 45 °C. Slides which had been previously fixed in methanol:acetic acid (3:1, v/v) for 10 minutes were coated with the diluted emulsion and incubated for 3 days at 4 °C. Autoradiographs were developed (Kodak D19 developer), fixed (Kodafix) and counterstained with Giemsa. All the labelled and unlabelled nuclei were counted in a defined area, at $\times 250$ magnification using a Zeiss R.A. microscope. The labelling index was then calculated, for each case, as the proportion of cells that had incorporated tritiated thymidine into DNA.

In the dose-response experiments 5, 10, 15, 20, 25 and 30 pieces of distal, posterior and anterior tissue were added to each well. Conditioned media were produced by culturing 5, 10, 20 and 30 pieces of distal, posterior and anterior of chick limb tissue in low serum medium for 24 h. The media were then centrifuged at 11 500 *g* for 5 mins and filtered (pore size 0.45 μm) before adding to the quiescent 3T3 cells. In later experiments lower concentrations of conditioned media (2.5, 5 & 10) were achieved by serially diluting conditioned medium made from 20 pieces of tissue. Variations between measurements caused by differential depletion of the medium, by the chick tissue pieces, were excluded by replacing the medium before labelling.

Grafting

Eggs were windowed at four days of incubation (stage 19–21) as before. Equivalent-sized pieces of distal, posterior and anterior tissue (of side approx. 200 μm) were dissected from donor embryos using tungsten needles and grafted into prepared anterior sites on host wing buds, opposite somite 16. Grafts were held in place by pins made from platinum wire (25 μm diameter; Goodfellow Metals, Cambridge, England). After operating, the eggs were sealed with Sellotape and incubated for a further seven days, by which time the cartilage elements had developed sufficiently to process the wings as whole mounts. Left and right wings were removed from the embryos and fixed in 5% aqueous trichloroacetic acid (v/v). The limbs were then stained with 0.1% (v/v) Alcian green in acid alcohol (1% concentrated hydrochloric acid in 70% aqueous alcohol, v/v), differentiated in acid alcohol, dehydrated and cleared in methyl salicylate (Summerbell & Wolpert, 1973).

RESULTS

Effect of different regions

Every type of tissue tested stimulated the quiescent 3T3 cells to initiate DNA synthesis. Posterior and distal tissues, however, were more effective than anterior or flank tissues, producing labelling indices of 50–60% compared to about 30%. In the absence of chick tissue, only about 15% of the cells became labelled. Using a Student's *t*-test for small samples, these results were found to be significantly different, at least at the 0.05 level of rejection (Table 1) and the same pattern of

Table 1. *Quiescent 3T3 cells were cocultured with 15 pieces of chick tissue for 24 h, followed by incubation with tritiated thymidine for a further 18 h.*

Tissue	Total cells counted	Number cells labelled	Labelling index (%)	Mean L. I. (%)
Anterior margin	18 880	5 870	31.1	29.0
	22 678	4 752	21.0	
	5 180	1 800	34.8	
Posterior margin	20 117	8 322	41.4	44.3
	21 692	9 722	44.8	
	5 873	2 734	46.6	
Distal tip	19 200	13 533	70.5	65.8
	17 573	10 694	60.9	
	4 832	3 182	65.9	
Flank	18 363	5 953	32.4	28.9
	3 638	924	25.4	
None	10 238	1 885	18.4	14.0
	21 138	2 283	10.4	
	4 751	609	12.8	

The labelling indices (L. I.) were then calculated as the proportion of nuclei that had incorporated labelled thymidine.

results was observed at all times. Initially the labelling indices were determined by counting approximately a quarter of the cells in each well (about 20 000 cells). However, it soon became apparent that this was more than required to show an effect, so between 2 and 5000 cells were counted in each case. When the test tissues were removed, some of the chick cells were found to adhere to the slides but these cells were excluded from subsequent counts. They were easy to identify, being considerably smaller than the mouse-derived 3T3 cells.

Effect of different amounts of tissue

Several attempts were made to measure accurately the number of chick cells added to each well. After removal from the wells, the chick tissues were dissociated with pronase and hyaluronidase or with trypsin and the cells counted using a haemocytometer. Total protein was also assayed following solubilization of the tissues in sodium hydroxide. However, no reliable method was found to account for the chick cells that had attached to the glass slides or for the mouse cells that had not. As an alternative approach, we varied the amount of tissue added to each well (Table 2). While no simple relationship was seen between dosage and the size of the response, it is unlikely that the results were influenced by small differences in the amount of tissue present. Labelling appears to be maximal at the lowest dose, while five pieces of posterior or distal tissue appear to produce higher labelling indices than thirty pieces of anterior tissue.

Table 2. The labelling indices of 3T3 cells following coculture with varying numbers of anterior, posterior and distal tissue pieces. The cells were labelled for 18 hours and the labelling indices calculated as before.

No. tissue pieces	Distal	Posterior	Anterior	Control
5	58.6	52.4	25.3	14.0
10	49.5	48.9	25.1	11.3
15	47.7	48.0	23.4	11.6
20	49.5	51.8	25.8	10.4
25	52.4	48.8	24.7	13.1
30	62.3	61.9	30.5	14.6

Table 3. The labelling indices of 3T3 cells following coculture with cell-free media, conditioned by incubating with varying amounts of anterior, posterior and distal tissue for 24 h. In all cases the cells were labelled for 18 h.

No. tissue pieces	Distal	Posterior	Anterior	Control
2.5	22.0	15.7	10.4	11.9
5	32.4	27.4	19.3	14.8
10	38.3	34.3	22.2	18.4
15	41.5	37.2	35.6	20.5
20	50.2	41.8	37.2	20.7
30	50.2	49.9	38.2	17.2

Effect of conditioned media

The conditioned media experiments were designed to show that contact between the chick tissue and the 3T3 cells was not a prerequisite for the stimulation of DNA synthesis (Table 3). Although the conditioned media clearly produced results comparable to those of the coculture experiments there were several important differences. The distinctions between the various tissues were less marked and the maximal responses with posterior and distal tissue were reduced. There also appeared to be a greater effect of dose. Taken together these results indicate that medium conditioned with chick tissue is not such a potent mitogen as the tissue itself. Interestingly, labelling with anterior conditioned medium was higher than with anterior tissue. It should also be noted that the level of labelling in the control wells was slightly higher in these later experiments.

Effect of grafting

The normal anteroposterior pattern of digits is 2, 3, 4, with digit 4 always forming closest to the polarizing region. Following a ZPA graft there may be six digits in the pattern 4, 3, 2, 2, 3, 4, but lesser reduplications also occur, such as 3, 2, 2, 3, 4 or

2, 2, 3, 4. Analysis of the grafting experiments revealed that while ZPA grafts always produced extra digits (11/11), neither distal (0/17) nor anterior (0/12) wing tissue were able to induce reduplications. The only additional structures that formed following distal transplantation were small cartilage nodules, probably due to self-differentiation of the graft.

DISCUSSION

Our results show that quiescent cultures of 3T3 cells can be stimulated to synthesize DNA by coculture with cubes of embryonic chick limb tissue. Distal and posterior regions, which have been shown to have morphogenetic activity during limb development, produce a greater response than non-morphogenetic regions, such as the anterior margin or the flank. There did not appear to be any substantial differences between the mitogenic properties of posterior and distal tissue. Although it is not generally considered that proximodistal development is chemically regulated, it is unlikely that diffusible morphogens produced by the posterior polarizing region and present in the distal mesoderm could enhance labelling to the same extent as the posterior tissue itself.

Previous *in vitro* experiments have shown that contact is not necessary for the ZPA or the AER to exert their effects on target cells (MacCabe & Parker, 1975; Calandra & MacCabe, 1978; MacCabe, Knouse & Richardson, 1981), which agree with our observations using conditioned media. This is consistent with the idea that diffusible substances secreted by the chick tissues are responsible for stimulating DNA synthesis in the 3T3 cells. While there is some evidence that the ZPA signals by producing a diffusible morphogen (Smith, Tickle & Wolpert, 1978; Honig, Smith, Hornbruch, & Wolpert, 1981; Tickle, 1981), the relationship between such a morphogen and the stimulating activity we describe here is not yet clear and would not account for the results seen with distal tissue.

In the dose-response experiments the absolute amount of chick tissue present did not appear to regulate the extent of the response. There was no consistent increase in labelling with increasing numbers of tissue pieces and a maximal response was achieved with only five pieces of tissue. Thus, variations between measurements could not be attributed to systematic errors in the amount of tissue added to each well. Conditioned media, on the other hand, did show a dose effect and a reduced maximal response, which indicates that media conditioned with chick tissues were not able to induce DNA synthesis to the same extent as the tissues themselves. This may be due to the degradation of mitogens during prolonged culture (Honig, 1983). Variations in labelling caused by the differential depletion of the medium by the chick cells were excluded by replacing the medium before labelling.

Two explanations can be offered for the effect of dose on response. Either all the regions of the limb are synthesizing the same or similar substances but in differing amounts, or the various regions could be making different substances. If the signal

was quantitatively different between regions, increases in labelling should be seen with increasing amounts of anterior tissue. Since the dose effect was relatively small, it may be that the differences between the regions are qualitative. Each signal could have its own level of response and the results could be due to interactions between them. A similar effect can be seen with growth factors *in vitro*, where epidermal growth factor and prostaglandin $F_{2\alpha}$ synergistically stimulate 3T3 cells to enter S phase (Jimenez de Asua, Richmond & Otto, 1981). Further complexity could arise if inhibitors as well as promoters of DNA synthesis were present together.

The grafting experiments confirm that distal tissue is unable to induce reduplications in the anteroposterior axis. This result was not unexpected as polarizing activity tends to be restricted to a fairly discrete area, at the posterior margin of the limb bud (MacCabe, Gasseling & Saunders, 1973; Summerbell & Honig, 1982). Since the proliferative effects of distal and posterior limb tissue on 3T3 cells appear to be very similar it may be that the controls for pattern and growth are not the same.

Further experiments using our present system will tell us considerably more about the mechanisms involved in growth control. Isolating molecules from conditioned media, however, may prove difficult if the substances produced by the chick cells were like growth factors and present in very low concentrations. Also, there exist several variants of 3T3 cells and using these, together with other cell lines, we hope to learn more about the nature of the molecules involved.

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