The preliminary characterization of mitogens secreted by embryonic chick wing bud tissues *in vitro*

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SUMMARY

Embryonic chick wing bud tissues secrete diffusible mitogens when cultured *in vitro* (Bell & McLachlan, 1985). These molecules may play an important role in limb development since media conditioned by morphogenetically active regions of the wing bud possess greater mitogenic activity than media conditioned by non-morphogenetic regions. These studies show that while the chick-derived growth factors were mitogenic for mouse-derived NIH 3T3, 10T1/2 and NR6 cells and chick limb bud cells, they did not stimulate DNA synthesis in 3B11, PC13 END, normal rat kidney or bovine endothelial cells. Furthermore, the effects of the chick-derived mitogens were synergistically enhanced by insulin and $PGF_{2\alpha}$ but remained unaffected by ECDGF, EGF, FGF and MSA. These findings indicate that embryonic chick limb bud cells synthesize and secrete growth factors which resemble in function other well-characterized growth factors and in particular PDGF.

INTRODUCTION

Two regions of the embryonic chick wing bud, the apical ectodermal ridge (AER) and the posterior zone of polarizing activity (ZPA), have been shown to alter the rate and pattern of growth during limb development *in vivo* (Saunders, 1948; Saunders & Gasseling, 1968; Saunders, Gasseling & Errick, 1976; Cooke & Summerbell, 1980; Bell, 1985). The ZPA and the AER have also been shown to influence the survival and growth of limb bud tissue explants *in vitro* in the absence of cell contact (MacCabe & Parker, 1975; Calandra & MacCabe, 1978; Solursh, Singley & Reiter, 1981; Reiter & Solursh, 1982). It has been suggested that both these regions influence pattern formation *via* the production of diffusible morphogens (Tickle, Summerbell & Wolpert, 1975; Meinhardt, 1983*a,b*). It is possible that the diffusion of a mitogen from a localized source could provide a positional signalling mechanism within the developing limb.

Since growth factors and their receptors have been isolated from a wide range of embryonic tissues, it is generally considered that they play an important role during development. In the chick embryo, for example, insulin-like growth factors (IGFs), insulin and epidermal growth factor (EGF) are all expressed early

in development (Gaspard, Klitgaard & Wondergem, 1981; De Pablo, Roth, Hernandez & Pruss, 1982; Mesiano, Browne & Thorburn, 1985). Similarly, embryo-derived cells have been shown to produce various growth factors when cultured *in vitro* (Gudas, Singh & Stiles, 1983; Rizzino, 1983; Heath & Isacke, 1984).

By stage 20 the chick limb bud is highly vascularized (Caplan & Koutroupas, 1973) and the circulation could provide the limb bud cells with a wide range of plasma- and platelet-derived growth factors in addition to any made by the limb bud cells themselves. It would be difficult to identify any mitogens produced by chick wing bud tissue and almost impossible to purify them using conventional methods because they appear to be present in such small amounts. As an alternative approach, the mitogenic effects of media conditioned by different regions of the chick limb bud were compared to those of several well-characterized growth factors using a simple coculture assay system in an attempt to find features in common. In addition to mouse-derived 3T3 cells, various other cell lines with differing growth factor requirements were also tested.

MATERIALS AND METHODS

Cell culture

Alpha-modified Eagle's medium (Alpha; Stanners, Eliceiri & Green, 1971) without added nucleosides or deoxynucleosides and Dulbecco's modified Eagle's medium (DMEM; Dulbecco & Freeman, 1959) purchased from Flow were supplemented with antibiotics (penicillin G, $75 \mu g \, ml^{-1}$ and streptomycin sulphate, $50 \mu g \, ml^{-1}$; Glaxo), L-glutamine, $29 \cdot 2 \mu g \, ml^{-1}$ (Sigma) and foetal calf serum (FCS; Sera-Labs). Cells were maintained as monolayer cultures at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in air, in Alpha containing 5% (v/v) FCS. Chick limb bud cells and endothelial cells were cultured in DMEM, also containing 5% (v/v) FCS.

Cell lines

NIH 3T3 cells (derived from Swiss 3T3 cells) were a gift from J. E. DeLarco, NIH, Bethesda, Maryland, USA (Todaro & Green, 1963). 10T1/2 were a fibroblast cell line derived from a C3H mouse embryo, from the Sir William Dunn School of Pathology, Oxford (Reznikoff, Brankow & Heidelburger, 1973). NRK cells were normal rat kidney fibroblasts, clone 49F, and were a gift from J. E. DeLarco (DeLarco & Todaro, 1978b). NR6 cells were derived from 3T3 cells and were a gift from H. Herschman, UCLA, Calif., USA (Pruss & Herschman, 1977). PC13 END cells were differentiated cells derived from PC13 embryonal carcinoma cells (PC13 EC) following retinoic acid treatment, and were a gift of J. K. Heath, Department of Zoology, Oxford (Heath & Deller, 1983). 3B11/1C cells were derived from 3T3 cells following transformation with Moloney murine sarcoma virus (MoSV40), and were a gift from J. E. DeLarco (DeLarco & Todaro, 1978a). Bovine aortic endothelial cells (ENDO) were isolated from adult aortae and were a gift of J. K. Heath (Gimbrone, Cotran & Folkman, 1976). Chick limb bud cells (CLB) were isolated from homogenized stage-19 to -21 chick limb buds (Hamburger & Hamilton, 1951).

Growth factors and conditioned media

Insulin (porcine) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were purchased from Sigma. Epidermal growth factor (EGF) was prepared from mouse salivary glands by the method of Savage & Cohen (1972) and was a gift of M. Gregoriou, Laboratory of Molecular Biophysics, Oxford. Embryonal carcinoma-derived growth factor (ECDGF), from EC cell-conditioned medium,

fibroblast growth factor (FGF), from bovine pituitary glands and multiplication-stimulating activity (MSA), from Buffalo rat liver cell-conditioned medium, were all purified using HPLC and were a gift of J. K. Heath. Platelet-derived growth factor (PDGF), prepared by the method of Johnsson, Heldin, Westermark & Wasteson (1982), was a gift of C-H. Heldin, Uppsala University, Sweden. PDGF was used at a concentration of 50 ng ml^{-1}, while in all other cases the final growth factor concentration was 20 ng ml^{-1}. Conditioned media were produced by culturing five pieces of either distal, posterior or anterior chick limb bud tissue in 0.5 ml of Alpha 0.5% FCS for 18 h (Bell & McLachlan, 1985). The media were then centrifuged at 1500 rev. min^{-1} for 5 min and filtered (pore size $0.45 \mu\text{m}$; Millipore) before use.

Growth factor and conditioned media assays

Cells were transferred to tissue culture chamber slides (Flow) at a concentration of $1-2 \times 10^4$ cells per well in 0.5 ml Alpha 5% FCS (or DMEM 5% FCS). The cells were allowed to attach and approach confluence before replacing the medium with Alpha 0.5% FCS (or DMEM 0.5% FCS). They were then left in low serum medium for at least 24 h to reduce the number of cell divisions occurring before the addition of any mitogens. Responding cells were incubated with conditioned media and/or growth factors for 6 h before being exposed to methyl-[^{3}H]thymidine (final concentration $1 \mu\text{Ci ml}^{-1}$; specific activity 40 Ci mmol^{-1}; Amersham International) for a further 18 h. After labelling, the plastic chambers were detached from the slides, the cells washed in PBS (Oxoid) and fixed in methanol: acetic acid (3:1; v/v) for 10 min.

Autoradiography and calculation of labelling indices

Slides were coated with emulsion (Nuclear Research Emulsion, K2, Ilford), mixed with an equal volume of an aqueous solution of glycerol (2% v/v) at 45°C, and exposed for 3 days at 4°C. Autoradiographs were developed in D19 (Kodak) and fixed (Kodafix) and the cells were counterstained with Giemsa (BDH). Between 1000 and 2000 labelled and unlabelled cells were counted in each case, at a magnification of ×250, using a Zeiss R. A. microscope. Labelling indices were then calculated as the proportion of nuclei that had incorporated tritiated thymidine.

RESULTS

Results of the growth factor studies

The results of the growth factor studies are shown in Table 1. Six growth factors were tested on six different cell lines; 3B11 cells did not respond to any of the growth factors; 3T3 cells responded to all the growth factors except insulin; 10T1/2 cells did not respond to $\text{PGF}_{2\alpha}$; endothelial cells only responded to EGF and FGF; NR6 cells did not respond to EGF, MSA or $\text{PGF}_{2\alpha}$; and NRK cells did not respond to insulin or $\text{PGF}_{2\alpha}$. Only a limited amount of PDGF was available, which when added to 3T3 cells produced a labelling index of 43.4%.

These cell lines, together with endothelial cells and chick limb bud cells, were then incubated in chick-tissue-conditioned media (Fig. 1). While 3T3, 10T1/2, NR6 cells and chick limb bud cells were stimulated to synthesize DNA following coculture with the chick-derived mitogens, 3B11, NRK, PC13 END and endothelial cells showed little or no response. In all cases in which there was a marked effect on the labelling index, media conditioned by distal and posterior chick wing bud tissue were more effective than medium conditioned by anterior tissue. The different types of chick-tissue-conditioned media were then added to quiescent cultures of 3T3 cells in combination with each of the six growth factors (Fig. 2).

The effects of ECDGF, FGF and MSA remained unchanged by the addition of conditioned media, whereas insulin and $PGF_{2\alpha}$ clearly enhanced the response to conditioned media and particularly the response to distal conditioned medium. The effect of EGF appeared to be slightly reduced by the presence of the chick-derived mitogens.

DISCUSSION

Different cell types differ in their response to growth factors because they express different sets of growth factor receptors. One way to test the biological activity of a putative growth factor is to examine the range of cell lines in which it stimulates DNA synthesis. A second way is to test whether it modulates the effects of other growth factors.

Primary cell lines such as bovine endothelial cells and chick limb bud cells have a limited lifespan in culture and a restricted response to growth factors, whereas 3B11 cells are transformed and as such do not require the presence of exogenous growth factors in order to proliferate. Established cell lines, such as 3T3, 10T1/2 and PC13 END cells, however, respond to a wide range of growth factors and exhibit density- and serum-dependent growth arrest.

Endothelial cells, which only respond to EGF, FGF and endothelial cell growth factor (ECGF; Maciag, Hoover & Weinstein, 1980), and PC13 END cells, which respond to EGF, FGF, insulin, IGFs and ECDGF (Heath, Bell & Rees, 1981; Heath & Isacke, 1984), did not respond to the chick-tissue-conditioned media. NR6 cells, which do not possess EGF receptors (Pruss & Herschman, 1977), responded to the chick-conditioned media to the same extent as 3T3 cells. Cell lines which did not respond to $PGF_{2\alpha}$, such as NR6, 3T3 and 10T1/2, did respond to the chick-derived growth factors. Also, the lack of response by NRK cells

Table 1. *Labelling indices of six cell lines following incubation with six different growth factors*

	3B11	3T3	10T1/2	ENDO	NR6	NRK
ECDGF	58·6	36·0	57·7	2·7	35·0	75·6
EGF	71·6	38·1	51·2	13·5	7·1	95·1
FGF	60·5	51·9	87·4	33·1	65·2	63·9
Insulin	49·1	18·7	26·8	2·9	24·5	18·4
MSA	61·1	26·1	24·5	1·1	5·2	44·3
$PGF_{2\alpha}$	42·5	23·6	8·6	0·2	4·6	16·1
None	65·4	12·4	7·6	2·1	6·7	14·5

In all cases growth factors were added to the cells, growing in low serum medium, at a final concentration of $20\,ng\,ml^{-1}$. After 6h the responding cells were exposed to tritiated thymidine for a further 18h. Labelling indices were calculated as the proportion of nuclei that had incorporated labelled thymidine. Experiments were repeated two or three times and the average standard deviation on the labelling indices was 5·8.
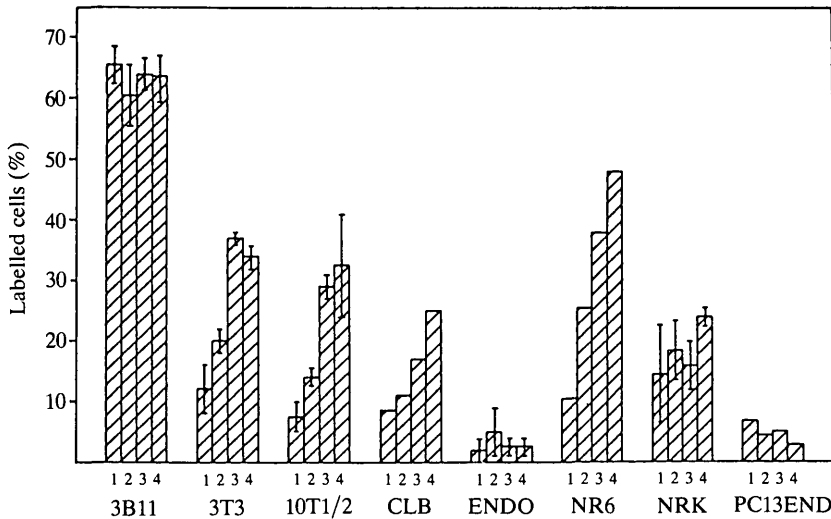
Fig. 1. The effects of conditioned media on different cell lines. Alpha medium supplemented with 0·5 % FCS was conditioned by five pieces of anterior (2), posterior (3) or distal (4) chick wing bud tissue for 18 h and added to quiescent cultures of eight different cell lines. After 6 h the cells were exposed to tritiated thymidine for a further 18 h. Control wells (1) contained only Alpha 0·5 % FCS. Error bars are shown where experiments were repeated.

suggests that the conditioned media do not contain transforming growth factors (TGFs), or that this activity is produced in very low amounts.

Under certain experimental conditions, two or more growth factors can interact with each other in characteristic ways to stimulate or inhibit the proliferation of responding cells synergistically (Rozengurt, 1982). Growth factors which act *via* the same receptor or by the same intracellular pathway are unlikely to synergize one another at maximal doses, e.g. TGF_α and EGF. Two growth factors which both stimulate mitosis in a target population of cells may in combination produce a greater response than with either growth factor alone. For example, PDGF and FGF both interact synergistically with insulin-like growth factors to stimulate DNA synthesis in quiescent cultures of 3T3 cells (Pledger, Stiles, Antoniades & Scher, 1978; Clemmons, Van Wyk & Pledger, 1980). Insulin, which has little mitogenic effect alone, synergistically enhances the effects of both $PGF_{2\alpha}$ and FGF (Otto, Ulrich & Jimenez de Asua, 1980).

In this study, the effects of the chick-tissue-conditioned media were synergistically enhanced by both insulin and $PGF_{2\alpha}$. The maximal response to chick-conditioned media is about 50 % (Bell & McLachlan, 1985), while the maximal response to insulin or $PGF_{2\alpha}$ is approximately 25 % (Otto *et al.* 1980). If the chick-cell-conditioned medium had contained either insulin or $PGF_{2\alpha}$, it is unlikely that it would have interacted synergistically with either of these two growth factors. The effect of EGF was slightly reduced by the presence of the chick-derived mitogens. This suggests that the conditioned media did not contain either insulin or ECDGF, which normally enhance the effects of both EGF and TGF_α (Salomon

et al. 1984). It also suggests that the chick-tissue-conditioned media contain PDGF which decreases the number of EGF receptors on quiescent 3T3 cells by about 50% (Wharton, Leof, Pledger & O'Keefe 1982). Low concentrations of PDGF have also been shown to enhance the effects of insulin (Heldin, Westermark & Wasteson, 1979).

The effects of distal- and posterior-conditioned media were not affected by ECDGF, FGF or MSA. This would occur if the chick-derived mitogens did not interact with any of these growth factors or if they affected the same processes in the target cells. In contrast, ECDGF and MSA appeared to inhibit the effects of anterior-conditioned medium (Fig. 2). Inhibition of growth factor activity may be caused by one growth factor modifying the binding of another to its receptor or by reducing the number of available receptors. This suggests that the chick cells secrete more than one growth factor, since mitogens produced by the anterior region do not appear to cooperate with other growth factors in the same way as posterior- and distal-derived mitogens.

In order for the chick mitogens to stimulate DNA synthesis in the responding cells, they must bind to specific receptors on the cell surface. This suggests that the chick-derived mitogens are structurally related to common growth factors and are as yet unidentified. Growth factors have been implicated in several developmental
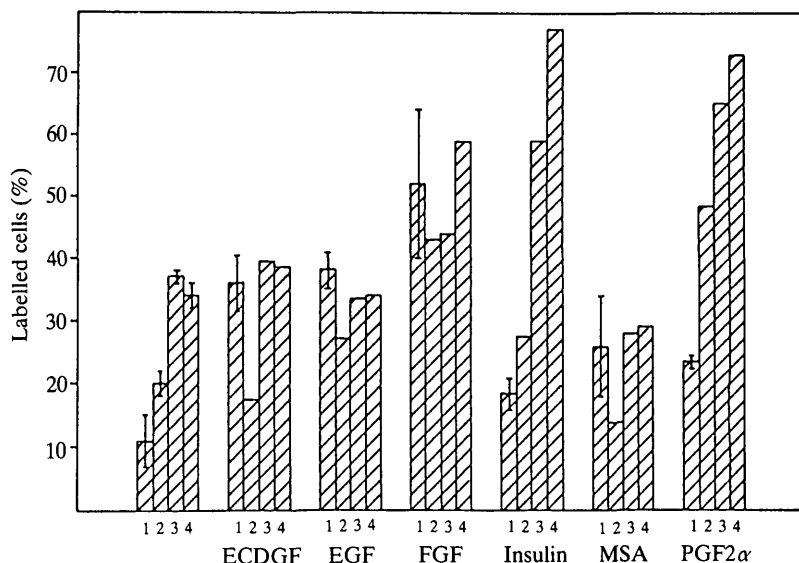

Fig. 2. Synergism between conditioned media and known growth factors. Alpha medium supplemented with 0·5% FCS was conditioned by five pieces of anterior (2), posterior (3) or distal (4) chick wing bud tissue for 18h and added to quiescent cultures of 3T3 cells in combination with one of six growth factors. After 6h, the responding cells were exposed to tritiated thymidine for a further 18h. Control wells (1) contained only Alpha 0·5% FCS plus one of the growth factors. The first set of results shows the labelling indices of cells exposed only to conditioned media or Alpha 0·5% FCS in the absence of any known growth factors. Error bars are shown where experiments were repeated.

systems and embryonic cells in culture have been shown to secrete growth regulatory molecules. Therefore, it is not surprising that embryonic chick wing bud cells also secrete mitogenic substances *in vitro*. Significantly, morphogenetically active regions of the wing bud possess greater mitogenic potential than non-morphogenetic regions, suggesting that growth factors may play an important role during limb development. While it is possible that the mitogens produced by the distal and posterior regions of the chick wing bud control growth in the embryonic chick wing, their relationship to pattern formation remains elusive. Also, it is not clear whether the different regions of the wing bud produce different amounts of the same growth factor or whether they synthesize and secrete different growth factors.

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