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PC13 embryonal carcinoma-derived growth factor

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A potent growth factor, PC13 embryonal carcinoma-derived growth factor (ECDGF), has been isolated from serum-free medium conditioned by PC13 murine embryonal carcinoma cells. ECDGF is a single chain, cationic hydrophobic molecule of 17 500 daltons. ECDGF will induce DNA synthesis in established fibroblast cell lines and the immediate differentiated progeny of PC13 EC cells *in vitro*, and consequently appears to differ from other well characterised growth factors both in structure and action.
Key words: growth factors/PC13 embryonal carcinoma/induction of proliferation

Introduction

Malignant PC13 embryonal carcinoma (EC) cells are derived from, and phenotypically resemble, the primitive ectoderm cells of the early mouse embryo. They may be induced, by treatment with retinoic acid, to undergo differentiation *in vitro* forming an apparently homogeneous cell type PC13 END (Rees *et al.*, 1979). Associated with this differentiation step is a significant change in the control of cell proliferation, including loss of the malignant phenotype (Rayner and Graham, 1982), acquisition of a finite proliferative lifespan *in vitro* (Rayner and Pulsford, 1984) and the appearance of a requirement for exogenous growth factors, such as epidermal growth factor (EGF, Rees *et al.*, 1979) and insulin (Heath *et al.*, 1981) to support cell proliferation. The developmental regulation of PC13 cell proliferation has consequently been the subject of investigation as a means to understanding the normal and abnormal control of cell multiplication in early mammalian embryogenesis (reviewed by Heath, 1983).

Our previous studies (Heath and Deller, 1983) revealed that there is no absolute requirement for a source of exogenous growth factors to support EC cell multiplication. There are in fact strong indications that EC cells themselves secrete growth regulatory molecules which can influence the growth of other cell types *in vitro*. Thus, Isacke and Deller (1983) observed that co-culture of both PC13 END cells and established fibroblast cells with PC13 EC cells resulted in the induction of heterologous cellular DNA synthesis. This effect is at least partly mediated by soluble, diffusible, factors released into the culture medium by PC13 EC cells. Similarly, Rizzino *et al.* (1983) reported that co-culture with EC cells could induce limited anchorage-independent growth of normal rat kidney (NRK) indicator cells in semi-solid media. Similar effects on the growth of NRK cells could be observed by exposure to PC13 EC cell conditioned serum free media (Heath, 1983). Gudas *et al.* (1983) further reported the existence, in medium conditioned by PSA1 EC cells, of activities which would reduce the binding of platelet-derived growth factor (PDGF) to its target cell receptor, and induce fibroblast DNA synthesis *in vitro*.

The identity of the agent(s) responsible for these effects is of interest, since the expression of endogenous growth factors by EC cells could partially explain the absence of a requirement for exogenous growth factors to support EC cell multiplication, and that growth factors secreted by EC cells would be candidates for mediating agents in the control of early embryonic growth *in vivo* (discussed by Heath, 1983). Here we report the purification of such a growth factor from serum-free medium conditioned by PC13 EC cells grown *in vitro*.

Results

General comments

Our preliminary characterisation of growth regulatory molecules expressed in PC13 EC cells focussed on gel filtration of lyophilised conditioned medium in 1 M acetic acid. This approach yielded a complex mixture of biologically active fractions, ranging in apparent mol. wt. from 20 000 daltons to 5000 daltons or less (Heath and Isacke, 1983). However, gel filtration of PC13 EC cell conditioned media in isotonic, neutral pH buffers in the presence of carrier proteins yielded a single peak of mitogenic activity of apparent mol. wt. 20 000 daltons. It was possible, therefore, that exposure of PC13 EC cell conditioned medium to acid conditions resulted in degradation of a primary 20 000 dalton species into active and inactive lower mol. wt. fractions. This phenomenon has been reported for other growth regulatory molecules such as endothelial cell growth factor (Maciag *et al.*, 1982). In addition, in these preliminary studies unacceptable losses of biological activity occurred when active fractions were concentrated by lyophilisation or ultrafiltration. Together these findings led to the development of procedures based on chromatographic methods of sample concentration employing neutral pH conditions in the preliminary phases of purification.

A second issue to be considered at the outset of this study was the necessity for the use of ECM serum-free medium (Heath and Deller, 1983) to support normal PC13 EC cell viability and multiplication. This medium is supplemented with plasma-derived substances which might be contaminated with trace amounts of mitogenic molecules. A serum-free medium (termed ECM/F, Heath and Isacke, 1983) was developed for C3H/10T1/2 mouse fibroblasts from the components of ECM media used to grow PC13 EC cells. The initiation of fibroblast DNA synthesis and cell division in medium ECM/F is dependent upon the presence of exogenous mitogens such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) (e.g., Figure 6). Growth promoting activity in experimental samples can consequently be detected by measuring the induction of

C3H/10T1/2 DNA synthesis in the absence of exogenous mitogens. If C3H/10T1/2 cells are plated into medium ECM/F previously conditioned by PC13 EC cells, up to 80% of the cells enter DNA synthesis within 24 h compared with 6% or less in the presence of unconditioned media components (Heath, 1983). The presence of mitogenic activity in PC13 EC cell-conditioned ECM medium is therefore dependent upon prior exposure to viable EC cells and is not a consequence of detectable contamination of media components by plasma-derived growth factors. This serum-free assay system provided a sensitive method for the specific detection of EC cell-derived growth factor and was employed in subsequent purification studies.

Purification of PC13 EC cell-derived growth factor

Pilot studies indicated that all the detectable mitogenic activity in PC13 EC cell-conditioned medium could be removed by exposure of conditioned media to cation ion-exchange matrices such as CM-Sephadex at physiological pH and ionic strength. Mitogenic activity could subsequently be recovered by elution with high ionic strength buffers at neutral pH (data not shown). The use of ion-exchange chromatography as a first step is relatively independent of starting sample volume, results in significant concentration of biological activity and the removal of all the deliberately added soluble media supplements.

The growth promoting activity present in the high salt eluate from the cation exchange step was found to bind to hydrophobic gel matrices such as phenyl-Sepharose. The mitogenic activity bound to phenyl-Sepharose was subjected to successive step-wise elution firstly with low ionic strength buffer and then with increasing concentrations of ethylene glycol. Growth promoting activity was eluted at 50% ethylene glycol as a single component (Figure 1a) suggesting the existence of an activity which interacts strongly with hydrophobic supports. It was found that biological activity was rapidly lost from the dilute aqueous fractions obtained from the ion-exchange step but was stable to storage (up to 1 month at 4°C) in the 50% ethylene glycol eluate from hydrophobic interaction chromatography. For routine purification therefore, ion-exchange and hydrophobic interaction chromatography were combined whereby the activity bound to CM-Sephadex was eluted by 1 M NaCl onto a column of phenyl-Sepharose which was subsequently washed with 40% ethylene glycol prior to elution of active components by 50% ethylene glycol. About half the biological activity present in conditioned medium was recovered after chromatography on CM-Sephadex and phenyl-Sepharose (Table I).

Further concentration and purification of growth promoting activity was achieved by trace enrichment of the phenyl-Sepharose eluate on disposable cartridges of macroparticulate octadecyl silica (ODS silica) at acid pH (Bennett *et al.*, 1978). The activity was recovered by step-wise elution with increasing concentrations of acetonitrile/0.1% trifluoroacetic acid (TFA), between 30% and 50% acetonitrile (Figure 1b). For routine purposes the cartridge was extensively washed with 20% acetonitrile/0.1% TFA before elution with 50% acetonitrile/0.1% TFA. This procedure results in modest yields: ~80% of the activity present in the eluate from phenyl-Sepharose chromatography is lost at this step (Table I). Reconstitution experiments indicated that this was a consequence of irreversible loss rather than the separation of two distinct interacting growth factor species. Trace enrichment does nevertheless result in significant sample concentration and increase in specific biological activity (Table I).

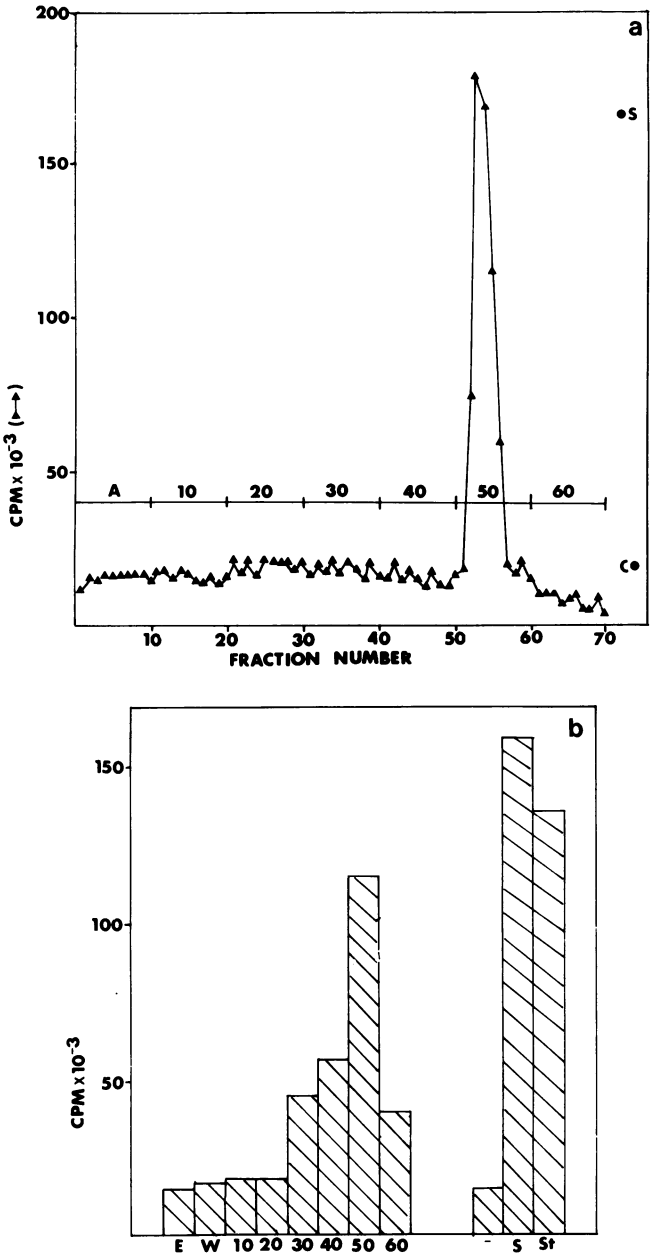

Fig. 1. (**a**) Chromatography of activity from CM-Sephadex chromatography on phenyl-Sepharose. Activity bound to CM-Sephadex was eluted by 1 M NaCl onto phenyl-Sepharose which was eluted with low-ionic strength buffer followed by increasing concentrations of ethylene glycol. 10 ml fractions were collected and 50 μl aliquots from each fraction tested for the induction of DNA synthesis in C3H/10T1/2 fibroblasts by [^{3}H]thymidine incorporation. A = buffer A (see Materials and methods), 10,20 etc. = concentration of ethylene glycol in buffer A (v/v). (**b**) Trace enrichment of growth-promoting activity eluted from phenyl-Sepharose by 50% ethylene glycol on ODS cartridge. 10 μl aliquots were removed from each fraction for the determination of biological activity as described in (**a**). E = unabsorbed material in eluate, W = material eluted by washing cartridge with 0.1% TFA, 10,20 etc. = concentration of acetonitrile in 0.1% TFA (v/v) used for step-wise elution. (–) = No additives, S = [^{3}H]thymidine incorporation induced by addition of 10% (v/v) FCS, St = activity present in 50 μl of starting material (50% ethylene glycol eluate, 60 ml total volume).

Samples from the trace-enrichment step were further concentrated by solvent evaporation and subjected to high-pressure size exclusion chromatography in 40% aceto-

Table I.

Step	Protein μg	U/ml	U/μg	Total U	Purification -fold	Yield %
1) Conditioned Media	6.3×10^5	1.2	9.7×10^{-3}	6100	1	100
CM-Sephadex/ Phenyl Sepharose[a]	–[b]	51.6	–	3100	–	50.8
3) Trace Enrichment	74	560	7.5	560	773	9.18
4) SEC Peak I	9.3	136	14.6	204	1505	3.34
Peak II	3.4	228	100.5	342	10 309	5.6
5) SEC Peak II	2.1	158	150.0	316	15 463	5.2

[a]Figures refer to combined ion-exchange and hydrophobic interaction chromatography.
[b]Not determined.

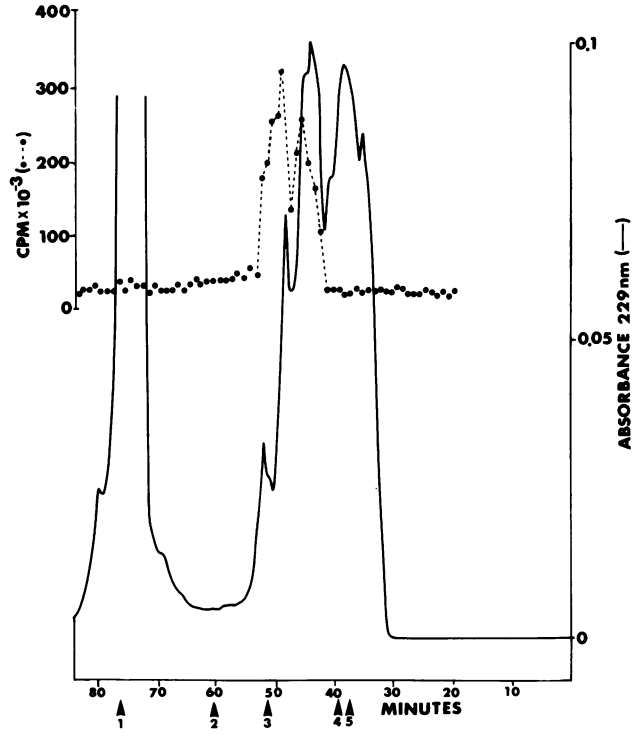

Fig. 2. High pressure SEC of growth-promoting activity recovered from trace enrichment step; flow-rate; 0.5 ml/min, fraction size; 0.5 ml. Growth promoting activity was determined as in Figure 1 using 10 μl aliquots from each column fraction. Elution positions of vitamin B12 (1), insulin (2), lysozyme (3), ovalbumin (4) and bovine serum albumin (5) under identical conditions are indicated below. Peak 1 fractions eluted between 45 and 47 min, peak 2 fractions eluted between 49 and 51 min.

nitrile/0.1% TFA (Bennett *et al.*, 1983). Growth promoting activity was separated into two peaks of 21 000 daltons (peak 1) and 17 500 daltons (peak 2) (Figure 2). The latter activity was of higher specific activity than the peak 1 material and was associated with a discrete absorbance peak which was separated from higher mol. wt. contaminants. Material in the peak 2 fractions was pooled, concentrated by solvent evaporation, and re-chromatographed under the same conditions. A single u.v. absorbance peak of 17 500 daltons, containing growth-promoting activity was observed (Figure 3). Peak material was dried by solvent evaporation and ~1 μg of

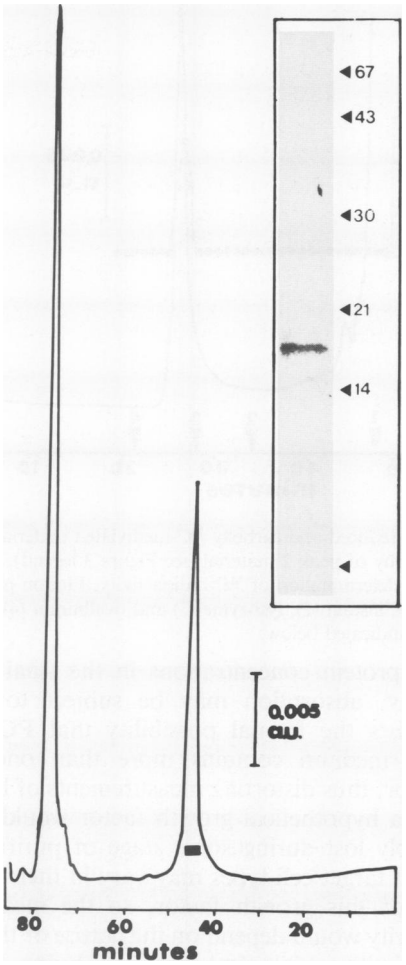

Fig. 3. SEC re-chromatography of peak 2 fraction; flow-rate; 0.5 ml/min. The eluate indicated by the bar (2 ml) was collected manually. **Inset**: approximately 1 μg of material recovered from re-chromatography was subjected to non-reducing SDS polyacrylamide gel electrophoresis followed by silver staining. Arrows indicate relative mobility of unreduced standards; bromophenol blue, lysozyme (14), soybean trypsin inhibitor (21), carbonic anhydrase (30), ovalbumin (43) and bovine serum albumin (67).

protein subjected to electrophoresis, under non-reducing conditions, in polyacrylamide gels containing SDS followed by silver staining (Figure 3 inset). A single protein species of 17 500 daltons was observed. It has not proved possible to recover biological activity from SDS gels after electrophoresis. However, since this species coincides with the homogeneous u.v. absorbance peak on SEC and has a high specific biological activity (Table I) before electrophoresis, it is probable that it represents homogeneous PC13 embryonal carcinoma-derived growth factor. We cannot, however, formally eliminate the possibility that the biological activity in this material is due to undetectable amounts of some material which is active at considerably lower concentrations and has very similar physical properties to the 17 500 dalton species detected by u.v. absorption and silver staining. No growth promoting activity could be recovered at any step from equivalent volumes of unconditioned ECM media fractionated by the methods described above. The existence of this 17 500 dalton species and associated growth-promoting activity in conditioned medium is therefore dependent upon exposure to viable EC cells.

Precise quantitation of the purification steps described above is complicated by several problems: (i) the estimation

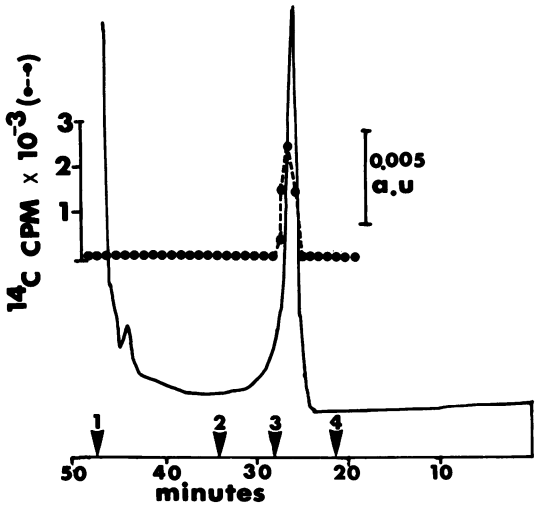

Fig. 4. SEC of reduced and carboxy ^{14}C-methylated material from rechromatography of peak 2 material (see Figure 3 legend). 50 μl aliquots were taken for determination of ^{14}C radioactivity. Elution positions of vitamin B12 (1), insulin (2), lysozyme (3) and ovalbumin (4) under identical conditions are indicated below.

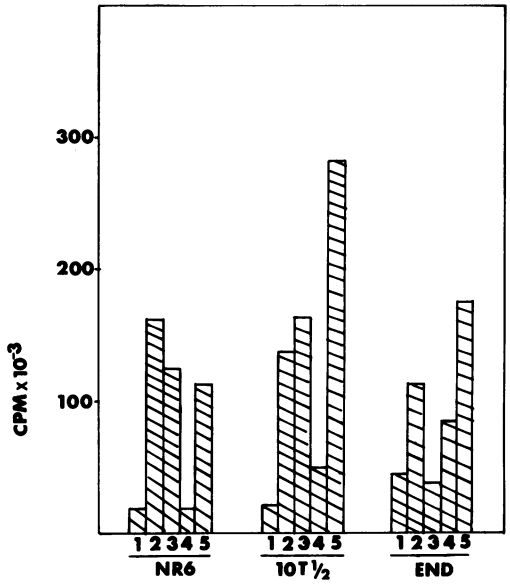

Fig. 5. Relative activities of ECDGF and growth factor standards determined by [^{3}H]thymidine incorporation in NR6.3T3, C3H/10T1/2 and PC13 END cells. Rat IGF-II (50 ng/ml) was substituted for insulin in medium ECM/F for the culture of C3H/10T1/2 cells in this experiment. 1 = no additions, 2 = 10% (v/v) FCS, 3 = 10 ng/ml PDGF, 4 = 50 ng/ml EGF, 5 = 10 ng/ml ECDGF.

of the low protein concentrations in the final purification steps by u.v. absorption may be subject to inaccuracy, (ii) there exists the formal possibility that PC13 EC cell-conditioned medium contains more than one species of growth factor, thus distorting measurements of biological activity (such a hypothetical growth factor would presumably be irreversibly lost during some stage of purification), and (iii) different target cell types may vary in their sensitivity to the action of this growth factor, so the measurement of specific activity would depend on the nature of the target cells used to monitor biological activity during purification. Allowing for these inaccuracies the overall yield of EC cell-derived growth factor in these experiments appears modest (Table I); ~5% of the biological activity present in the starting conditioned medium is recovered at the second SEC step. The material purified as peak 2 described above, and associated with a 17 500 dalton protein species [henceforth termed EC cell-derived growth factor (ECDGF)] is, however, comparable in potency with purified PDGF as a mitogen for C3H/10T1/2 cells under our assay conditions (Table I).

Characterisation of PC13 EC cell-derived growth factor

It is important to establish if PC13 ECDGF is related to other well characterised growth factors such as EGF, PDGF and IGF-II. Reduction and radioactive alkylation of PC13 ECDGF yields a single peak of coincident radioactivity and u.v. absorption (Figure 4) suggesting that PC13 ECDGF is a single chain molecule containing reactive cysteine groups following reduction. There would appear to be a difference in structure, therefore, between the material purified from EC cell-conditioned medium and PDGF (which is a 30 000 dalton, two chain molecule, Heldin *et al.*, 1981) or EGF and IGF-II, which are both of lower apparent mol. wt. under identical chromatographic conditions. Similar considerations would suggest that ECDGF also differs from human platelet derived β-transforming growth factor (two 12 500 dalton subunits, Assoian *et al.*, 1983), and human melanoma-derived, or feline sarcoma virus-transformed rat cell-derived, EGF-like transforming growth factors (one 7400 dalton chain, Marquardt and Todaro, 1982; Massague, 1983). Nevertheless, a rigorous test of the existence of possible structural relationships with these, and other less well characterised growth factors, awaits the determination of appropriate amino acid sequence data.

As an alternative approach to this issue, it is possible to compare the variety of cell types which are susceptible to the growth promoting action of PC13 ECDGF and other growth factors. Thus PC13 ECDGF appears to differ from EGF (and presumably other growth factors which depend on the EGF receptor to exert their action) in that it will induce DNA synthesis in EGF receptor deficient NR6.3T3 mouse fibroblasts (Figure 5). PC13 ECDGF also appears to differ from PDGF in that it will induce DNA synthesis in PC13 END cells whereas PDGF is inactive (Figure 5). PC13 ECDGF is also mitogenic for C3H10T 1/2 mouse fibroblasts which do not respond to IGF-II by significant induction of DNA synthesis (Figure 5). On these grounds also, PC13 ECDGF differs from PDGF, EGF and IGF-II.

Discussion

Here we report the isolation and preliminary characterisation of a potent growth factor from ECM serum-free medium conditioned by PC13 murine EC cells. ECDGF is a cationic, hydrophobic, single chain molecule of apparent mol. wt. 17 500 daltons. It appears to differ significantly from other well characterised growth factors both in structure and in its variety of susceptible target cells.

The size heterogeneity in activity we have observed in high resolution SEC suggests that the 17 500 dalton ECDGF species may be derived from a larger biologically active form. It will be necessary to purify the activity present in peak 1 fractions to test this hypothesis. However, the existence of biologically active growth factor sequences in longer precursor transcripts (e.g., Jansen *et al.*, 1983) and the existence of related growth factors of different mol. wts. (e.g., Marquardt *et al.*, 1980) has been observed in other cases. Furthermore, the production of multiple active species under acid solubilisation conditions (Heath and Isacke, 1983) suggests that the capacity for further artifactual or natural processing

of ECDGF into biologically active fractions may exist.

We have shown that ECDGF, although sharing with PDGF a common capacity to induce the proliferation of established fibroblast cell lines, differs both in structure and in its capacity to induce the proliferation of PC13 END cells. We have also failed to detect the expression, by PC13 EC cells, of either PDGF-related v-*sis* (Waterfield *et al.*, 1983) transcripts (unpublished observations of J.K. Heath, R. Hill and N. Hastie) or biologically active substances which react with antibodies raised against purified PDGF (unpublished observations of J.K. Heath). These findings contrast with a report of the secretion of PDGF-related growth factors from PSA1 EC cells (Gudas *et al.*, 1983). There are several possible explanations for this apparent discrepancy. Firstly, it is possible that different established EC cell lines may differ in the growth factors they secrete. There are precedents for the existence of functional differences between different EC cell lines in that insulin receptors are expressed by F9 EC cells (Nagarajan and Anderson, 1982) but not by PC13 EC cells (Heath *et al.*, 1981). Secondly, it is possible that ECDGF may affect the expression of fibroblast PDGF receptors by transmodulation (Rozengurt *et al.*, 1981) and may share some common post-receptor intracellular pathways with PDGF. This would account for the ability of PSA1 conditioned medium to reduce the binding of radiolabelled PDGF to target cells and the expression of PDGF-inducible transcripts in cells exposed to PSA1 conditioned medium (Cochran *et al.*, 1983). Stern and Priddle (1984) have further identified a growth promoting activity, apparent mol. wt. 6000 daltons, in medium conditioned by subclones of the PC13 EC cells employed here which were selected for their capacity to survive and proliferate in reduced concentrations of foetal calf serum (Stern and Heath, 1983). It is possible that this activity represents a biologically active fragment of the 17 500 dalton species secreted by the parental cell line or may again be an entirely different growth factor species. Since the recovery of biological activity in our experiments is low, it is not possible to eliminate stringently the possibility that PC13 EC cells secrete more than one growth factor species. The availability of purified preparations of ECDGF will, however, allow its relatedness to these various other growth promoting activities to be determined.

The origin of EC cells from the primitive ectoderm of the early post-implantation mouse embryo (Diwan and Stevens, 1976), and their close phenotypic similarity with primitive ectoderm cells (Evans *et al.*, 1979) implies that ECDGF has a role in the regulation of cell proliferation in the early post-implantation embryo. It is significant, therefore, that ECDGF will induce the proliferation of PC13 END cells which are the immediate differentiated progeny of PC13 EC cells. The expression of ECDGF by PC13 EC cells may in fact account for part of the co-operative growth phenomenon observed to exist between EC cells and END cells *in vitro*, and thought to be related to growth control processes occurring in normal embryogenesis (Isacke and Deller, 1983). Partially purified preparations of ECDGF will also support the proliferation of specific primary embryonic cell types *in vitro* (J.K. Heath and A. Wills, in preparation). However, ECDGF may not be restricted in its action to the early embryo since it will also induce the proliferation of foetus-derived fibroblast cell lines. It is possible therefore that multiple sites of ECDGF synthesis exist both in the embryo and foetus and that ECDGF regulates cell proliferation in both embryonic and foetal development *in vivo*. It will clearly be informative therefore to localise sites of ECDGF expression in the embryo and foetus.

Materials and methods

Cells

PC13 clone JD and PC13 clone 1A4 were used between passages 4 and 25. The origin and maintainance of these clones has been described (Heath and Deller, 1983). EGF receptor-deficient NR6.3T3 derivatives from mouse 3T3 fibroblasts (Pruss and Herschman, 1977) were generously provided by Dr. H. Herschman (University of California at Los Angeles). C3H/10T1/2 mouse fibroblasts (Reznikoff *et al.*, 1973) were obtained from I.M. Williams, Sir William Dunn School of Pathology, Oxford and were subcultured according to the '3T3' regime of Todaro and Green (1963). Cells were discarded after 25 passages from the original stock (passage level unknown). PC13 END cells were obtained by retinoic acid treatment of PC13 EC cells as previously described (Heath and Deller, 1983).

Growth factors and media supplements

Purified platelet-derived growth factor (Heldin *et al.*, 1981) was a generous gift of Dr. C.-H. Heldin, Uppsala. Purified murine epidermal growth factor was a gift of Dr. M. Gregoriou, Department of Molecular Biophysics, University of Oxford. Human plasma fibronectin was a gift of Dr. J. Smith, Blood transfusion service plasma fractionation laboratory, Churchill Hospital, Oxford. Iron-conjugated transferrin, insulin, rat IGF-II (MSA) and human plasma lipoproteins were prepared as previously described (Heath and Deller, 1983).

Cell culture

All tissue culture plastic was obtained from Nunc (through Gibco, UK). Foetal calf serum (FCS) was selected batches from Gibco (UK) and Sera-Labs (UK). Trypsin was obtained through Difco (UK). Dulbecco's modified Eagles medium (DME) and Hams medium F12 were obtained as dry powder from Flow (UK) and prepared according to manufacturer's instructions.

Procedures for routine and serum-free culture of PC13 EC cells were as described previously (Heath and Deller, 1983). PC13 EC cell-conditioned ECM medium was prepared as follows: PC13 EC cells were plated directly into 175 cm^2 gelatin- and fibronectin-coated tissue culture flasks containing 250 ml of gas phase and temperature-equilibrated ECM medium [prepared with a basal medium of DME:F12 (50:50 v/v) supplemented with 5 μg/ml transferrin, 50 μg/ml LDL and 50 μg/ml HDL] at a density of 5×10^5 cells per flask. The medium was collected after 4 days culture, by which time the cells had reached confluence and were discarded. Conditioned medium was centrifuged at 9000 *g* for 30 min to remove cells and debris and used immediately.

Biological assays

C3H/10T1/2, PC13 END and NR6.3T3 cells were removed from stock culture flasks by the low temperature trypsinisation procedure described by McKeehan (1977) using 0.125% trypsin, 10 mM EDTA in Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS). The cells were pelleted by low speed centrifugation, resuspended in DME:F12 (50:50) and plated at a density of 5×10^4 cells per well into gelatin- and fibronectin-coated 24-well cluster dishes containing 2 ml of pre-equilibrated medium ECM/F per well. ECM/F was prepared as for ECM medium in a basal medium of DME:F12 (50:50) and additionally supplemented with porcine insulin (100 ng/ml) or rat IGF-II (50 ng/ml). The cells were cultured in a fully humidified gas phase of 5% CO_2 in air at 37°C. Test samples were added after 24 h culture, followed by 1 μCi of [^{3}H]thymidine ([^{3}H]methyl thymidine, 40 mCi/mmol Amersham) 6 h later. The cells were cultured for a further 18 h before processing for scintillation counting. The cell monolayers were washed with PBS and fixed for 15 min with 1 ml/well methanol:glacial acetic acid (3:1 v/v) at room temperature. Fixed cells were air-dried and then incubated at 4°C with two successive changes of 10% trichloroacetic acid (30 min each incubation). The cells were then washed with 2 ml of distilled water and incubated for 2 h at 37°C in 250 μl 0.2 N NaOH per well. Solubilised material was dissolved in 2 ml Aqualumma plus (LKB, Croydon) and counted on a RackBeta scintillation counter (LKB, Croydon) at an efficiency of 34%. [^{3}H]Thymidine incorporation into PC13 END cells was as above, except that the cells were used for experiments following 5 days exposure to retinoic acid and replated into 24 well plates containing 2 ml/well DME:F12 supplemented with 5% (by volume) FCS. After 24 h culture the medium was changed to 2 ml/well ECM medium and test sample additions made 24 h later.

The growth promoting activity in specific sample fractions for quantitation of purification procedures was measured by titration of activity on C3H/10T1/2 cells and comparison with that of a purified PDGF reference stock. 1 unit of activity was defined as that which gave equivalent [^{3}H]-thymidine incorporation (in c.p.m.) into C3H/10T1/2 cells as 10 ng/ml PDGF.

Chromatographic procedures

Ion-exchange chromatography. Conditioned medium was subjected to ion-exchange chromatography in 5 litre batches. CM-Sephadex C50 (Pharmacia, UK) was swollen in buffer A (50 mM sodium phosphate, 150 mM NaCl, 5 mM EDTA, pH 7.2) overnight at 4°C. 100 ml of gel slurry was added to each litre of conditioned medium and stirred overnight at 4°C. The gel was collected by filtration on a sintered glass funnel and washed with 5 litres of buffer A to remove unbound components. The washed gel was packed into a 5 cm x 50 cm column and washed with a further 500 ml of Buffer A. The ion-exchange column was then eluted at 4°C at flow-rate of 35 ml/h with 600 ml of buffer B (50 mM sodium phosphate, 1 M NaCl, 5 mM EDTA, pH 7.2) at 4°C collecting 15 ml fractions into disposable polypropylene tubes.

Hydrophobic interaction chromatography. 7 mls of phenyl-C1 Sepharose (Pharmacia, UK) was extensively washed with 50% propanol in distilled water followed by 50 volumes of Buffer A, packed into a 2.4 cm diameter x 1.5 cm glass column and further equilibrated by washing with 50 ml buffer B. The phenyl-Sepharose column was then either connected to the outlet of the ion-exchange column, which was then eluted through the phenyl-Sepharose column with buffer B as above, or pooled ion-exchange fractions were passed onto the column at 4°C at a flow-rate of 35 ml/h. The column was then stepwise eluted at 4°C with 100 ml of buffer A followed by 100 ml each of increasing concentrations of ethylene glycol (Aldrich Chemicals, UK) in buffer A at a flow-rate of 20 ml/h, collecting 10 ml fractions.

Trace enrichment. Sep-Pak C18 cartridges (ODS cartridge, Waters, UK) were activated by washing with 20 ml acetonitrile (Rathburn Chemicals, UK, h.p.l.c. grade) followed by 20 ml 0.1% TFA (sequencer grade, Rathburn Chemicals, UK) in water ('analar' grade, BDH, UK). Pooled fractions from the phenyl-Sepharose column eluate were adjusted to pH 2.3 by addition of neat TFA and passed twice through the ODS cartridge, which was then washed with 20 ml of 0.1% TFA, followed by back-flushing with successive 2 ml portions of increasing concentrations of acetonitrile/0.1% TFA dissolved in 0.1% TFA/water. The second ml of each eluate was collected for testing biological activity. For routine purification, the ODS cartridge was washed with 20 ml 20% acetonitrile:0.1% TFA in water and active fractions eluted by back-flushing with two successive 1 ml portions of 50% acetonitrile:0.1% TFA in water. Biological activity was recovered in the second ml of the eluate.

High pressure liquid chromatography. H.p.l.c. apparatus comprised two Altex model 112 pumps, Altex model 421 controller, Altex 340 organiser and model 210 sample injection valve, a Beckman model 160 fixed wave length detector fitted with 229 nm filters and a Kipp and Zonen BD-41 dual channel pen chart recorder (all through Beckman, UK). H.p.l.c. size exclusion chromatography (SEC) was performed with a 7.5 mm x 8 cm TSK-SW3000 precolumn (LKB, UK), and 7.5 mm x 30 cm TSK-G4000SW (Beckman, UK) and 7.5 mm x 60 cm TSK-G3000SW (LKB, UK) SEC columns connected in series in a solvent of 0.1% TFA, 40% acetonitrile (h.p.l.c. grade S, Rathburn Chemicals, UK) in water ('analar', BHD, UK) at ambient temperature at a flow-rate of 0.5 ml/min. Sample fractions from trace enrichment were concentrated to ~100 μl by solvent evaporation under a stream of helium gas at room temperature before injection. Fractions were collected at 1 min intervals with an appropriate time delay to synchronise fraction collection with detector events. Fractions from SEC were concentrated to ~200 μl by solvent evaporation as above before re-chromatography as above.

SDS polyacrylamide gel electrophoresis. Samples from the second SEC step were freeze dried and then subjected to electrophoresis in discontinuous polyacrylamide gels using the SDS containing buffer system of Laemmli (1970) with resolving gels containing 15% acrylamide. The gels were then silver stained by the method of Ansorge (1983). Mol. wt. standards were 1 μg each of lysozyme (14 000 daltons), soybean trypsin inhibitor (21 000 daltons), carbonic anhydrase (30 000 daltons), ovalbumin (42 000 daltons) and bovine serum albumin (67 000 daltons). The lower detection limit of this method is ~20 ng of a single protein species.

Reduction and carboxyl ^{14}C-methylation. This was performed by a modification of the method of Christie and Gagnon (1982). Samples were evaporated to dryness under a stream of helium and re-dissolved in 200 μl 6 M guanadinium hydrochloride, 0.5 M Tris-HCl, 2 mM EDTA, 50 mM dithiothreitol pH 8.2 and incubated under helium at 37°C for 3 h. Iodo[2-^{14}C]acetic acid (50 μCi:57 mCi/mmol, Amersham, UK) was added, the mixture was incubated for 5 min at room temperature, unlabelled iodoacetic acid was added to a final concentration of 200 mM and the mixture was incubated for 30 min on ice. The reduced and alkylated sample was subjected to SEC on a 7.5 mm x 60 cm TSK-G2000SW (Altex) column in 40% acetonitrile/0.1% TFA at a flow-rate of 0.5 ml/min at ambient temperature. 0.5 ml fractions were collected and 50 μl aliquots dissolved in 2 ml scintillation fluid for the determination of radioactivity.

Protein quantitation. Protein was determined by the dye binding method of Read and Northcote (1981) using bovine serum albumin as standards. Conditioned medium was desalted on a 10 ml column of Sephadex G-25 in PBS to remove coloured interfering materials in basal medium. The protein concentration in the eluate from phenyl-Sepharose column was not determined due to interference from ethylene glycol in the dye binding reaction. Aliquots from the trace enrichment step were freeze dried and reconstituted directly in the dye solution. Protein concentration in pooled fractions from the h.p.l.c. steps was estimated from the absorbance at 280 nm assuming an absorbance of 1 at 280 nm for 1 mg/ml protein concentration.

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