ACQUISITION OF A LIMITED LIFESPAN BY DIFFERENTIATING CELLS DERIVED FROM PC13 EMBRYONAL CARCINOMA CELLS

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SUMMARY

Retinoic acid (RA) has previously been shown to induce the differentiation of mouse embryonal carcinoma (EC) cells to endoderm-like cells that have a slower rate of proliferation and are non-tumorigenic. These cells also acquire the ability to respond to a range of exogenous growth factors. We have analysed the change in growth phenotype for PC13 EC cells using video recordings and autoradiography. We have shown that the endoderm-like cells have a longer cell cycle time than their undifferentiated counterparts (five cell divisions after exposure to RA the differentiated cells had a median cell cycle time of 1800 min compared to 800 min for control cells). The endoderm-like cells also have a progressively decreasing probability of dividing again and this indicates that the differentiation process is accompanied by the acquisition of a limited life-span. The characteristics of mortal cells are well documented, and the endoderm-like cells demonstrate the properties of such cells.

In addition, we have confirmed the observation that epidermal growth factor (EGF) can stimulate the proliferation of the endoderm-like cells and have shown, using autoradiography, that 92% of these cells express EGF receptors. Using video recordings, we have demonstrated that the effect of EGF is to shorten the cell cycle of the differentiating cells. We have also shown that EGF can enhance the survival of the endoderm-like cells and thereby prolong their life-span. It is known that EGF and other growth factors can prolong the life-span of mortal cells derived from normal tissues, but we have demonstrated that EGF can have this effect on the differentiated derivatives of a tumour cell.

INTRODUCTION

The limited life-span of normal mammalian cells in culture is well documented (Hayflick & Moorhead, 1961; Absher, Absher & Barnes, 1974). There is also evidence that normal cells can be serially transplanted in vivo for only a limited period (Daniel, Aidells, Medina & Faulkin, 1975; Hellman, Botnick, Hannon & Vigneulle, 1978). In contrast, under favourable growth conditions, tumour cells exhibit unlimited proliferative potential (immortality) both in vitro and in vivo (Rheinwald & Beckett, 1981; Newbold, Overell & Connell, 1982; Newbold & Overell, 1983; and review by Ponten, 1976). The unlimited growth of tumour cells can be regulated in some instances, for example, mouse embryonal carcinoma (EC) cells, the stem cells of teratocarcinomas (Kleinsmith & Pierce, 1964), can be induced to differentiate in vitro and in vivo into non-tumorigenic endoderm-like cells (reviews by Graham, 1977; Hogan, Barlow & Tilly, 1983; Gardner, 1983). EC cell differentiation has generally

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been used as a model system to study early mouse development, including the regulation of cell proliferation during embryogenesis (reviews by Graham, 1977; Hogan et al. 1983), but the system also provides a useful tool for investigating differences in the properties of tumorigenic and non-tumorigenic cells (review by Heath, 1983).

PC13, and other EC cell lines, can be induced to form endoderm-like cells by exposure to retinoic acid (RA) (Strickland & Mahdavi, 1978; Adamson, Gaunt & Graham, 1979) and other retinoids (Jetten & Jetten, 1979). It has previously been shown that these endoderm-like cells are derived directly from undifferentiated cells rather than by selection of cells already present in the EC cell population. It was also shown that the endoderm-like cells proliferate more slowly than the EC cells and that this new growth phenotype, once attained, is stable: EC cells exposed to RA for longer than 24 h are usually committed to the differentiation of cells with a slower rate of proliferation (Rayner & Graham, 1982). In this paper we examine the change in growth phenotype in more detail: our main aim is to investigate the life-span of the two cell types. EC cells can be passaged indefinitely either in vitro or in vivo (Iles, 1977; review by Stevens 1983), but it has proved possible to derive permanent cell lines of their differentiated derivatives only by the application of strong selective pressure for a rapidly growing cell type (Lehman, Speers, Swartzendruber & Pierce, 1974; Solter, Shevinsky, Knowles & Strickland, 1979). It has been suggested that the endoderm-like cells formed in response to RA normally have a limited capacity to proliferate (Heath, 1983). We investigate this hypothesis using a combination of clonal analysis and video recordings. We show that, whilst PC13 EC cells have a constant rate of cell division and rarely die, the endoderm-like cell divide progressively more and more slowly and become increasingly less likely to divide again, i.e. they acquire a limited life-span. Use of video recordings has allowed us to quantify the change in growth phenotype.

Several EC cell lines (including PC13) can be serially cultured in serum-free medium (Rizzino & Sato, 1978; Heath & Deller, 1983). The growth of these cells may therefore be independent of exogenous growth factors. The endoderm-like cells, however, do not proliferate in serum-free medium (Isacke & Deller, 1983) and are able to respond to certain exogenous growth factors. Epidermal growth factor (EGF) (Rees, Adamson & Graham, 1979; Rizzino, Orme & DeLarco, 1983), insulin (Heath, Bell & Rees, 1981), sarcoma growth factor (Rizzino et al. 1983) and a high molecular weight factor produced by EC cells (Isacke & Deller, 1983) are all able to stimulate the proliferation of the endoderm-like cells. Here we examine the action of one of these growth factors, EGF, on the growth of the endoderm-like cells. We have used video recordings to study the effect of EGF on the cell cycle time and the life-span of these cells and, by the use of autoradiography, we have investigated their expression of specific receptors for EGF.

MATERIALS AND METHODS

All the experiments reported here were performed on a subclone derived from PC13 clone 5 EC cells (Bernstine, Hooper, Grandchamp & Ephrussi, 1973; Adamson et al. 1979). The subclone (MA2) was derived and cultured routinely as described previously (Rayner & Graham, 1982).
Effect of culture conditions on the growth of clones of EC cells

In order to investigate the growth of PC13 EC cells and their differentiated derivatives under various culture conditions we have used a clonal technique, fully described previously (Rayner & Graham, 1982). Briefly, single mitotic EC cells were individually plated into gelatin-coated 50 mm tissue culture dishes within a 6 × 8 grid, and then exposed to a test medium. Clones derived from all the cells that became attached were observed at daily intervals using a Wild M40 inverted-phase microscope. Clone growth was measured by counting the number of cells comprising each surviving clone.

Eight different culture media were tested for their effect on clone growth: (a) Alpha modified Eagle's medium (α medium) (Stanners, Elicieri & Green, 1971, without the addition of nucleosides or deoxynucleosides; Flow Laboratories, Irvine, Scotland) supplemented with 10% (v/v) foetal calf serum (FCS) (Sera-Lab, Crawley, England). FCS was heat-inactivated at 56 °C for 30 min. (b) α Medium with 10% FCS and containing $3 \times 10^{-7}$ M-retinoic acid (RA), the RA having been first dissolved in dimethyl sulphoxide (DMSO) at $3 \times 10^{-4}$ M. DMSO alone at this concentration has no effect on the growth of clones. (c) α Medium with 10% FCS and containing $3 \times 10^{-10}$ M epidermal growth factor (EGF) (culture grade; Collaborative Research, Waltham, Mass., U.S.A.). (d) α Medium with 10% FCS and containing $3 \times 10^{-7}$ M-RA and $3 \times 10^{-10}$ M-EGF. (e) α Medium with 0.5% FCS. (f) α Medium with 0.5% FCS and containing $3 \times 10^{-7}$ M-RA. (g) α Medium with 0.5% FCS and containing $3 \times 10^{-10}$ M-EGF. (h) α Medium with 0.5% FCS and containing $3 \times 10^{-7}$ M-RA and $3 \times 10^{-10}$ M-EGF.

Video recording of clone growth under different culture conditions

Preparation of clones before filming. Clones of cells growing in media (a), (b) and (d) were prepared for filming as described above, except that the EC cells were plated into gelatin-coated 25 cm$^2$ tissue culture flasks (Nunc, Paisley, Scotland) rather than into dishes. Although fewer cells could be plated into a flask than into a dish (a 6 × 3 grid was used), flasks could more easily be made air-tight. After the cells had attached and the test medium had been allowed to equilibrate (at 37 °C, in an atmosphere of 5% (v/v) CO$_2$ in air, for approximately 1 h) the flask was sealed by smearing the inside of the lid with a thick layer of high-vacuum grease (Dow Corning S.A., Belgium) and screwing it down tightly. Single clones were then filmed. Control clones, treated identically, but not filmed, were inspected microscopically (daily for 9 days) to assess whether the process of filming affected clone growth and survival. Clones growing without RA were filmed for 3 days (thereafter individual cells could not easily be distinguished), and clones growing with RA were filmed for 6 days. After the filming period, clones were inspected daily until 9 days after the start of the experiment.

All the video data presented here are derived from experiments in which the cloning efficiency of cells plated in the flask that was subsequently filmed was >75%. (For definition of cloning efficiency see legend to Fig. 2.) The process of filming clones, in these experiments, had no effect on their viability: 12/13 (92%) of filmed clones survived for at least 9 days and 83% ± 4.3% (mean ± S.E.M.; 13 experiments) of clones cultured in control (unfilmed) flasks survived for at least 9 days. Filming of clones also had no effect on their growth (data not shown). A total of 5.0% of cells cultured with RA and 0.01% of cells cultured without RA appeared to fuse with their sister cells. These cells have been excluded from all further calculations.

Recording apparatus. Clone growth was filmed using a video camera (National Panasonic WV-1350 AE/B), a time-lapse video recorder (Panasonic VTR-/NV 8030) and a monitor (Sony PVM-2000CE) attached to a Wild M40 inverted phase-contrast microscope located in a 37 °C controlled-temperature room. Light intensity at the camera during the recording was measured at 0.1 lux. Recording took place on a 80 h programme and playback on a 1 h programme. Event times were taken from tape revolutions and converted to real time by means of a calibration curve.

Analysis of tapes. The cloning procedure before filming ensured that all the cells on the monitor screen at any one time were the progeny of a single EC cell. After every 50 tape revolutions a tracing of the cells on screen was made on clear perspex plastic (50 tape revolutions = 190 min at beginning of tape, 490 min at end of tape). Photocopies of these tracings were cut into individual cells and the silhouettes weighed to provide a measure of cell area. A haemocytometer slide was filmed using the same recording apparatus and procedure in order to convert silhouette weights into real cell areas.
Cell division was indicated by a sudden rounding of the cell followed by the appearance of a division furrow. The completion of furrow formation (probably corresponding to telophase) could be scored with a reproducible accuracy of within one tape revolution (i.e. ±6 min) and was therefore the point from which cell age was measured. Cell death was indicated by a sudden rounding of the cell followed by detachment from the substratum and lysis. The sudden rounding of the cell could be scored with a reproducible accuracy of within one tape revolution and was therefore the point at which cell death was recorded.

Autoradiography to detect presence of EGF receptors

We used an autoradiographic method to examine EGF receptor expression by the endoderm-like cells. Autoradiography has the advantage of providing information about the percentage of EGF receptor-positive cells within the differentiated cell population and also about the range of expression of receptors amongst positive cells.

Seven-day-old clones of undifferentiated and differentiated cells were gently washed twice with 'binding buffer': Earle's balanced salt solution (Earle, 1943; Flow Laboratories, Irvine, Scotland) containing $5 \times 10^{-3} \text{M}-N-2$-hydroxyethylpiperazine-$N'$-2-ethanesulphonic acid (HEPES) and $0.4\%$ (w/v) bovine serum albumin (pH 7.4). After washing, the cells were exposed to 2 ml of $8.3 \times 10^{-11} \text{M}$-$^{125}\text{I}$-labelled EGF diluted in 'binding buffer' ($3.4 \times 10^5$ cts/min per ml), with or without a large excess of unlabelled EGF (approximately $1000 \times$ the concentration of $^{125}\text{I}$-labelled EGF). The $^{125}\text{I}$-labelled EGF, a gift from A. R. Rees, was prepared as described by Rees et al. (1979). The cells were incubated at room temperature for 2 h with intermittent shaking and then washed four times in ice-cold 'binding buffer'. The incubation temperature was chosen to minimize the proportion of receptors internalized during the incubation period, whilst allowing equilibrium binding, between EGF and its receptor, to be attained.

The cells were then fixed in methanol/acetic acid vapour (3:1, v:v) for 15 min and dried for 30 min at room temperature. This procedure ensured that the morphology of the cells was retained throughout further processing. After drying, the cells were exposed to $2\%$ (v/v) glutaraldehyde (BDH, Poole, England) in phosphate-buffered saline (PBS, solution 'A' of Dulbecco & Vogt, 1954) (pH 7.4) for 30 min at room temperature. Excess activated groups were quenched with two 15-min washes of $2\%$ glycine solution in PBS (pH 7.4). This procedure ensured that specifically bound EGF molecules were linked covalently with membrane molecules.

After drying overnight at room temperature each tissue culture dish was coated with photographic emulsion (Nuclear L4 emulsion; Ilford Research, Basildon, Essex, England) diluted 1:1 with distilled water and maintained at 50°C. The plates were dried overnight and stored at 4°C for 4–6 weeks. Autoradiographs were developed (D19 developer: Kodak, Hemel Hempstead, Herts, England; 2 min), rinsed in tap water, fixed (Kodafix; Kodak; 5 min), rinsed in running tap water and stained with Giemsa stain (BDH; pH 6.5) for 15 min at room temperature.

RESULTS

Effect of RA on clone growth

Video recordings of clone growth clearly illustrate the change in morphology that occurs when PC13 EC cells are stimulated to differentiate. Fig. 1 shows that the differentiating cells become progressively larger and more irregular in shape. Measurements of (projected) cell areas, derived from weighing their silhouettes, confirm this impression. Cells cultured in medium without RA remain small: $0.63 \times 10^{-3} \text{mm}^2 \pm 0.05 \times 10^{-3} \text{mm}^2$ (mean ± s.e.m. for cells of the second cell cycle after plating, $n = 24$). In contrast, cells cultured in medium with RA become progressively larger, attaining a maximum cell area, 4–6 days after plating, of $2.87 \times 10^{-3} \text{mm}^2 \pm 0.23 \times 10^{-3} \text{mm}^2$ (mean ± s.e.m., $n = 29$), i.e. $4.6 \times$ that of untreated cells.

Fig. 2 shows the effect of RA on the size of clones of EC cells. Whilst EC cells in
Fig. 1. Effect of culture conditions on cell morphology. Clones growing in α medium (+10% FCS) without RA (A), and with RA (B), were filmed using a video camera, a time-lapse video recorder and a monitor attached to an inverted phase-contrast microscope located in a 37°C controlled-temperature room. Consecutive panels represent what may be seen of the clone on the monitor screen at intervals of approx. 12 h after plating (×85 of actual cell size). Exact times are shown at the top left of each panel, in days and hours.

the absence of RA gave rise to clones that increase exponentially in cell number, clones of EC cells exposed to RA cease to grow after 3–5 days (10% FCS) or 1–2 days (0.5% FCS). There are several possible explanations for the change in the rate of clonal expansion that occurs in response to RA. The cell cycle of the differentiated cells could be longer; the cells could have a greater tendency to withdraw from the cell cycle; the cells could have an increased probability of dying. We have used video recordings to investigate these alternatives.

From video recordings of clone growth we constructed α plots (Smith & Martin, 1973; Brooks, Bennett & Smith, 1980; Peto et al. 1977) of cell cycle times for
successive generations of cells within clones. Incomplete cell cycle measurements resulting from cell death, movement of a cell out of the field of view and termination of recording were dealt with as described by Peto et al. (1977). Fig. 3 shows the $\alpha$ plots for the second to seventh cell cycle after plating for cells of clones cultured in the presence of RA. It shows that the cell cycles of the differentiating cells become progressively longer. Median cell cycle times for cells cultured both with and without RA were calculated from $\alpha$ plots and are shown in Fig. 4A. In contrast to the differentiating cells, the EC cells cultured without RA retain a relatively constant cell cycle time throughout the filming period. After five cell divisions, the median duration of the cell cycle of RA-treated cells had increased from 710 min to 1800 min, whilst the cell cycle of untreated cells had increased only slightly, from 720 min to 800 min.

Fig. 4B shows that after four cell cycles in the presence of RA, the differentiating cells have an increased probability of dying and in consequence a reduced probability

![Log mean clone size vs. days after plating](image1)

![Log mean clone size vs. days after plating](image2)

Fig. 2. Effect of culture conditions on the growth of clones of EC cells. A. Clones cultured in $\alpha$ medium + 10% FCS. (●●●) Clones cultured in $\alpha$ medium alone (−RA, −EGF; plating efficiency (PE): 44/48, cloning efficiency (CE): 86%). (◯◯◯) Clones cultured in $\alpha$ medium containing 3.3 $\times$ 10$^{-10}$M-EGF (−RA, +EGF), PE: 34/48, CE: 72%. (▼▼▼) Clones cultured in $\alpha$ medium containing 3 $\times$ 10$^{-7}$M-RA (+RA, −EGF), PE: 40/48, CE: 53%. (▽▽▽) Clones cultured in $\alpha$ medium containing RA and EGF (+RA, +EGF), PE: 41/48, CE: 76%. B. Clones cultured in $\alpha$ medium containing 0.5% FCS. (●●●) −RA, −EGF, PE: 40/48, CE: 68%. (◯◯◯) −RA, +EGF, PE: 38/48, CE: 30%. (▼▼▼) +RA, −EGF, PE: 39/48, CE: 3%. (▽▽▽) +RA, +EGF, PE: 36/48, CE: 8%. PE = no. of cells that attach to the substratum within 1 h of plating/ no. of single cells plated within grid squares. CE = no. of cells that give rise to clones surviving for 9 days after plating/ no. of cells that attach. Clones derived from cells that attached within 1 h of plating were inspected daily. The number of cells in each viable clone was counted to determine its 'clone size'. Points show the mean clone size of all clones that result from the cells that attach in a single experiment. Similar results were obtained in three separate experiments.
of dividing again. After six cell divisions in RA, only 55% of cells succeed in dividing again. In contrast, only a small percentage of the undifferentiated cells fail to divide (<10% in any one cell cycle). In other words, the differentiating cells, in contrast to their undifferentiated counterparts, have a limited proliferative life-span. The endoderm-like cells, which fail to divide, survive for much longer before dying than the few undifferentiated cells that die. The median survival time for all non-dividing RA-treated cells, calculated from an α plot, was 1800 min, which is significantly

Fig. 3. Effect of RA on cell cycle time of EC cells. α plots for cells of the 2nd to 7th cell cycles after initial exposure to RA. No. of clones analysed: 4. No. of cells analysed: 16, 32, 50, 96, 136, 96 for the 2nd to 7th cell cycles, respectively.

Fig. 4. Effect of culture conditions on intermitotic time (a) and mortality (b) of cells. Single EC cells were plated within flasks, exposed to α medium (+10% FCS) −RA −EGF (●●●), +RA −EGF (▼▼▼), +RA +EGF (▽▽▽) and filmed using a video camera. Total numbers of cells analysed: 12, 22, 78, 150, 294 (−RA −EGF; 6 clones); 8, 16, 32, 50, 96, 136, 96 (+RA −EGF; 4 clones); 6, 12, 20, 32, 54, 60, 44 (+RA +EGF; 3 clones) for cells of the first and subsequent cell cycles after plating, respectively. a. Median intermitotic times derived from α plots. b. % Mortality calculated from cells that remain on screen, i.e. are observed either to die or to divide.
longer than the median survival time for undifferentiated cells that died (640 min, $P < 0.05$, log Rank test).

**Effect of EGF on clone growth**

Since it has been demonstrated that certain growth factors can stimulate the proliferation of the differentiated derivatives of EC cells, we proceeded to study the effect of EGF on the duration of the cell cycle and the life-span of these cells. Fig. 5 shows that the majority of the endoderm-like cells (92%) expressed detectable EGF receptors, whereas their undifferentiated counterparts did not. Fig. 2 shows that

![Graphs showing the effect of EGF on clone growth.](image_url)
clones of undifferentiated cells exposed to EGF came to be significantly larger than their unexposed counterparts (at both concentrations of FCS used). Undifferentiated EC cells do not express EGF receptors, and consequently it is not surprising that they fail to respond to this growth factor. Fig. 2 also shows that the rate of clonal expansion of EGF-treated clones of undifferentiated cells slows with time. This indicates that EGF is effective in stimulating the proliferation of the endoderm-like cells only for a limited period during the process of their differentiation.

Video analysis of the clonal growth of differentiating cells with and without EGF shows that exposure to this growth factor ultimately results in a reduction in the cell cycle time of the endoderm-like cells (Fig. 4A). The median duration of the cell cycle of differentiated cells (six cell divisions after initial exposure to RA) stimulated with EGF was 1720 min. This is significantly shorter than that of unstimulated cells (3700 min, \( P < 0.05 \), log Rank test). Initially the presence of EGF acts to lengthen the cell cycle of the differentiating cells. This inhibitory effect of EGF on cell division may be related to the retarding effect of EGF on the clone growth of undifferentiated cells, which can be observed particularly at low serum concentrations (Fig. 2).

EGF also increases the probability of survival of endoderm-like cells (Fig. 4B). There is considerable variation in the eventual size of clones of endoderm-like cells (Rayner & Graham, 1982). Table 1, therefore, shows cell survival probabilities for cells of small and large clones separately. As might be expected, cell death was more extensive within small clones, but EGF improves cell survival within both small and large clones. Non-dividing endoderm-like cells also survived for longer when EGF was present: i.e. they had a median survival time of 2600 min in the presence of EGF as opposed to 1800 min when EGF was absent (\( P < 0.05 \), log Rank test).

**DISCUSSION**

In this study we have used video recordings to analyse the growth of PC13 EC cells and their differentiated derivatives *in vitro*. We have investigated properties of the two cell types that may be related to their capacity for growth *in vivo*. It had previously been shown that clones of the endoderm-like cells (4 days exposure to RA) are unable to form tumours when transplanted to a suitable site in a syngeneic host, whilst 87% of their untreated counterparts are able to do so (Rayner & Graham, 1982).
We have shown that the endoderm-like cells divide more slowly than the EC cells. This confirms the conclusion drawn from observations using relatively crude methods of measuring cell proliferation (Strickland & Mahdavi, 1978; Evain, Binet & Anderson, 1981; Linder, Krondahl, Sennerstam & Ringertz, 1981; Rosenstrauss, Sundell & Liskay, 1982; Rayner & Graham, 1982). We have been able to quantify this change in the rate of cell division in terms of the length of the cell cycle.

It is not self-evident that the non-tumorigenic endoderm-like cells should divide more slowly than the tumour cells from which they are derived. Many tumour cells divide no faster than normal cells and many divide more slowly (Steel, 1972; Risser & Pollack, 1974). Tumours normally increase in size faster than the surrounding tissue because a greater proportion of the constituent cells remain capable of division. The EC cells, as well as having a shorter cell cycle than the endoderm-like cells, are more likely to divide and less likely to die. A total of 99% of EC cells, of the sixth cell cycle after plating, divide again, whilst only 55% of RA-treated cells do so (Fig. 4B). This difference in the growth fraction of EC cells and their differentiated derivatives may be fundamental to the difference in their ability to form tumours.

It was observed some 25 years ago (Swim & Parker, 1957; Hayflick & Moorhead, 1961) that normal cells will continue to proliferate for only a limited number of passages in vitro. After a defined number of population doublings, characteristic of species and cell type, cultures of normal cells will usually cease to reproduce. In contrast, transformed and tumour cell cultures may be passaged indefinitely and seem to be immortal (review by Ponten, 1976). Video analyses of mortal cells suggest that they have both a progressively increasing cell cycle time and an increasing probability of withdrawal from the cell cycle (Absher et al., 1974; Belle et al., 1978). The differentiating EC cells, observed here, exhibit a similar growth phenotype, and this indicates that differentiation of PC13 EC cells to a non-tumorigenic cell type is associated with the acquisition of a limited lifespan.

The molecular basis of the mortality of normal cells is unknown. Two hypotheses to explain the phenomenon have been widely canvassed. The first suggests that a combination of autocatalytic error propagation in the translation of proteins and the standard experimental tissue culture procedures combine to kill off the cultures (Kirkwood & Holliday, 1975). The second supposes that mortality is an ordered, genetically controlled differentiation phenomenon (Shall & Stein, 1979). Here we have shown that PC13 EC cells exposed to RA acquire a limited life-span. The differentiation of EC cells to an endoderm-like cell type in response to RA is well documented and numerous changes in gene expression have been described (review by Hogan et al., 1983). It might be expected that the life-span of the endoderm-like cells, in common with the expression of new molecules, is also genetically regulated. We have shown that the loss of the capacity to divide, during differentiation, is both predictable and progressive and this observation can also be taken to support the argument that the restricted life-span of the endoderm-like cells is genetically controlled rather than a tissue-culture artifact (see Ponten, Stein & Shall, 1983).

It has often been implied that the endoderm-like cells, generated from EC cells in response to RA, are a homogeneous population of cells; although it has also frequently
been noted that these cells may express a range of phenotypes (Solter & Knowles, 1979; Knowles et al. 1980; Stern, Gilbert, Heath & Furth, 1982; Ogiso, Kume, Nishimune & Matsushiro, 1982). We have shown here that EC cells exposed to RA progressively divide more and more slowly, and that the differentiating cells become increasingly less likely to divide again. In other words, EC cells, in the process of acquiring a limited life-span, express a different growth phenotype with each round of cell division. This expression of a range of phenotypes during differentiation is also apparent from the analysis of the change in cell morphology. RA-treated cells can be morphologically distinguished from their untreated counterparts within 48 h exposure, but they attain their maximum size after three to four more divisions (Fig. 1).

Video analysis also showed that with each succeeding round of cell division the intermitotic times of RA-treated cells become significantly more variable. The coefficient of variation for cells of the second cell cycle after plating was 17% and for the sixth 59% \( (P < 0.01, F \) test; coefficients of variation were calculated from the \( \alpha \) plots shown in Fig. 3; Nachtewey & Cameron, 1968). Increasing variability of intermitotic times has been observed for other cell types which have a limited lifespan (Absher et al. 1974; Bell et al. 1978).

It has previously been demonstrated that several growth factors can stimulate the proliferation of the endoderm-like cells. In this paper we have examined the action of EGF on the differentiating cells in the light of our observation that they have a limited lifespan. Rees et al. (1979) had shown that at least some of the differentiating cells express EGF receptors within 24 h of exposure to RA. We have shown, using an autoradiographic method that gives information about receptor expression at the single-cell level, that 92% of the endoderm-like cells express detectable EGF receptors and should therefore be capable of a growth response.

We have demonstrated that EGF stimulates both a shortening of the cell-cycle time of the endoderm-like cells and also an increase in their probability of dividing again. In other words, EGF can prolong the life-span of these cells. It has been recorded that growth factors, including EGF, can prolong the life-span of inherently mortal cells (Rheinwald & Green, 1977; and see Hauschka et al. 1983), but to our knowledge this is the first demonstration that a growth factor can prolong the life-span of the non-tumorigenic differentiated derivatives of a tumour cell.

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Subsequent to the submission of this paper for publication, it has been brought to our attention that similar results to those presented here have been obtained by C. L. Mummery, C. E. van den Brink, P. T. van der Saag & S. W. de Laat (1984).

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Embryonal carcinoma cell differentiation


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