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Placental alkaline phosphatase activity is inversely related to cell growth rate in HeLaS3 cervical cancer cells

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Placental alkaline phosphatase is an inducible enzyme, expressed in HeLaS3 cells, which has been shown to possess protein phosphotyrosine phosphatase activity. Since phosphotyrosine levels are known to increase in actively dividing cells we sought an inverse correlation between PLAP activity and growth rate in HeLaS3 cells. We found that PLAP inducers, Na-butyrate, dexamethasone, bromodeoxyuridine and dibutyryl cAMP caused a dose-dependent reduction in growth rate. Mimosine, an agent that blocks the cell cycle in G1, caused an increase in PLAP activity whilst the mitogen EGF caused a corresponding decrease in PLAP activity. PLAP activity may therefore be related to cell proliferation rate

Placental alkaline phosphatase; Cell growth; Epidermal growth factor; Mimosine; G1 phase

1. INTRODUCTION

The fact that alkaline phosphatase is synthesized so ubiquitously in nature, by bacteria, plants and animals, indicates that this enzyme is involved in fundamental biochemical processes, but its physiological role is not yet clear [1]. Alkaline phosphatases (EC 3.1.3.1) comprise a group of enzymes which are classified by their ability to participate in hydrolase/transferase reactions on a wide range of phosphate containing compounds at pH 10–10.5 [1].

In man there are four different alkaline phosphatase genes; liver/kidney/bone (tissue-unspecific), intestinal, placental and germ cell (placental-like) [2], each of which is expressed in its own tissue-specific pattern. Alkaline phosphatase has been found in several organelles in various species, although in vertebrates it is primarily a plasma membrane enzyme [1]. The enzyme has been implicated in mammalian bone calcification [3] and phosphate transfer in the epithelial cells of the intestine [4], but the evidence for these roles of the enzyme is not conclusive. The lack of substrate specificity and the broad spectrum of activities exhibited by alkaline phosphatase has rendered the elucidation of the physiological function of the enzyme very difficult.

Over the past few years evidence has accumulated which suggests that alkaline phosphatase may possess phosphotyrosine phosphatase activity and thus may play a role in cell division in both normal and transformed cells [5]. It has been demonstrated that a variety of alkaline phosphatase preparations dephosphorylate phosphotyrosyl-histones, at physiological pH, at 5–10 times the rate observed with phosphoseryl-histones [6]. These enzymes were also active against phosphotyrosyl-proteins from A431 cells [6]. It has also been shown that the protein phosphatase activity of liver membranes is inhibited by levamisole (a specific alkaline phosphatase inhibitor) [7]. These results were interpreted as showing that alkaline phosphatase is the major protein phosphatase in liver membranes. Further, it has been shown that the characteristics of the enzyme responsible for protein phospohatase activity in bone cells correlated with those of bone cell alkaline phosphatase [8].

In the past is has been shown that in certain human cancer cell lines, which express alkaline phosphatase activity at low levels, this activity could be increased by exposure to a diverse group of agents with no apparent similarities in their chemical structure. These alkaline phosphatase inducers include glucocorticoids [9], sodium butyrate [9], bromodeoxyuridine [10] and dibutyryl cAMP [11]. One such cell line is the HeLaS3 cervical cancer cell line. This cell line synthesizes both placental and intestinal alkaline phosphatase [11,12]. Butyrate and prednisolone (a glucocorticoid) induce PLAP activity in these cells [9] and it has also been shown that these compounds arrest the cell cycle in G1 [11,13]. In light of evidence of a role for alkaline phosphatase as a tyrosine protein phosphatase and the fact that compounds which increase alkaline phosphatase activity may also decrease cell growth rate we decided to deter-

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Abbreviations. EGF, epidermal growth factor; FCS, foetal calf serum; MEM, minimal essential medium; PLAP, placental alkaline phosphatase

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Figure 1 Effect of butyrate on placental alkaline phosphatase activity and cell growth rate of HeLaS3 cells.

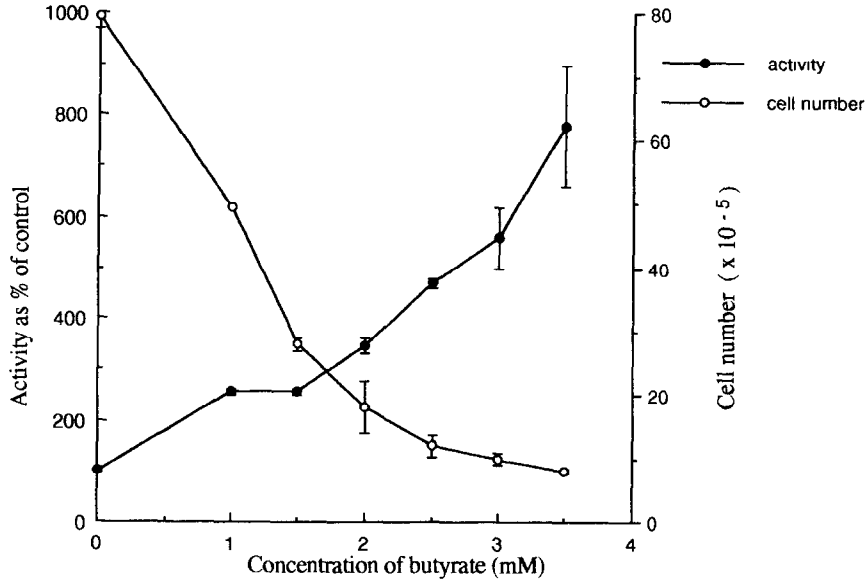

Fig. 1 HeLaS3 cells were seeded into 75 cm^2 flasks at approx. 9×10^5 cells/flask and allowed to attach over a 24 h period. They were then grown, either in the absence (control) or presence of butyrate, at the indicated concentrations, for 96 h. Cells were then harvested for cell counting and for assay of heat-stable alkaline phosphatase activity. The medium was changed every 24 h. The data represent mean ± S.E.M. of three experiments.

mine whether there is an inverse correlation between PLAP activity and cell growth rate in HeLaS3 cells.

2. MATERIALS AND METHODS

2.1. *Materials*

Minimum Essential Medium (MEM) with Earle's salts and penicillin/streptomycin was obtained from Gibco/BRL (UK). Foetal calf serum was obtained from Imperial Laboratories (UK). All other chemicals were obtained from Sigma Chemical Co. except for mimosine which was obtained from Aldrich Chemical Co.

2.2. *Cell culture*

HeLaS3 cells were grown in Minimum Essential Medium (MEM) with Earle's salts supplemented with 10% foetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% carbon dioxide/95% air.

2.3. *Induction procedures*

Cells were seeded into 75 cm^2 flasks at the indicated densities and allowed to attach for 24 h before addition of inducing agents. Cell counts were made with an Improved Neubauer haemocytometer, on trypsinised cells in complete medium. Cell viability was determined by trypan blue exclusion

2.4. *Enzyme assay*

Cell monolayers were washed three times with ice-cold PBS (0.14 M NaCl, 9 mM NaH_2PO_4, 3 mM KCl, 2 mM KH_2PO_4; pH 7.2) and detached into PBS using a rubber policeman. The cells were pelleted by centrifugation at 1000 × *g* for 5 min and the pellet was resuspended in 0.25% (w/v) sodium deoxycholate, pH 8.0. Alkaline phosphatase activity was measured by the release of phenol from phenyl phosphate at pH 10.7 at 37°C. Protein was determined using a Lowry protein determination kit. Enzyme activities are presented as % of control activity (where untreated cell activity was taken to be 100%).

2.5 *Growth of cells in serum free medium*

Cell monolayers were washed three times with PBS prior to transfer to MEM supplemented with BSA (0.2% v/v), transferrin (10 μg/ml), sodium selenite (2.6 ng/ml) and antibiotics, at previously described concentrations. Cells were grown in this medium for 24 h after which time they were grown either in the absence or presence of EGF (20 ng/ml).

3. RESULTS

3.1. *Heat stability of alkaline phosphatase in HeLaS3 cells*

HeLaS3 cells synthesize two alkaline phosphatase isoenzymes, PLAP and intestinal alkaline phosphatase [11,12]. In the past there has been no indication whether intestinal alkaline phosphatase is inducible in these cells. We decided to study the correlation between PLAP activity and cell growth since this isoenzyme is documented as being inducible by butyrate and by a synthetic glucocorticoid, prednisolone [9]. We initially characterized the heat stability at 65°C of alkaline phosphatase activity in our cells, this being an established method for distinguishing between heat-stable PLAP and heat-labile intestinal alkaline phosphatase activity.

Figure 2 Effect of dexamethasone on placental alkaline phosphatase activity and cell growth rate of HeLaS3 cells.

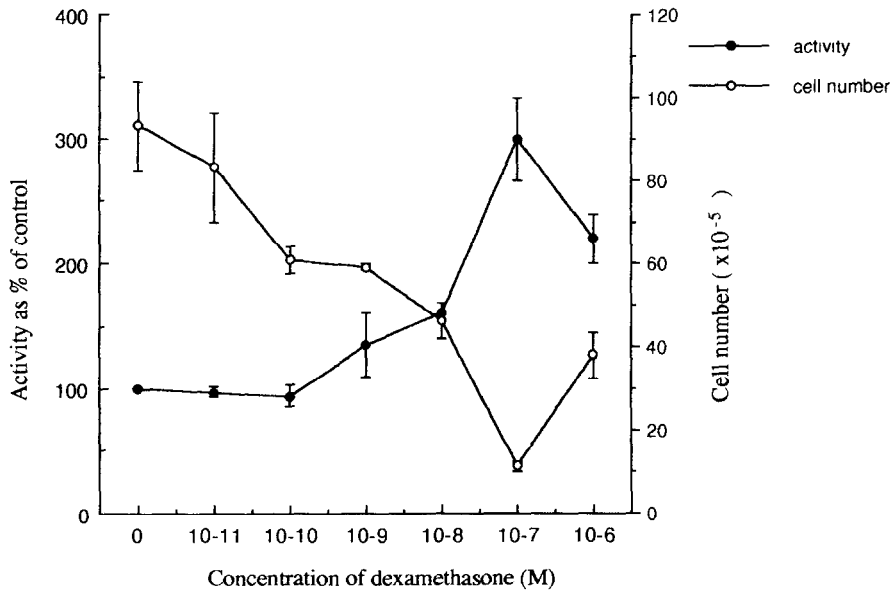

Fig. 2. HeLaS3 cells were seeded into 75 cm^2 flasks at approx 1×10^6 cells/flask and allowed to attach over a 24 h period. They were then grown, either in MEM supplemented with 4% of steroid-stripped newborn calf serum and 0.1% ethanol (control), or in medium containing the indicated concentrations of dexamethasone for 96 h. Cells were then harvested for cell counting and for assay of heat-stable alkaline phosphatase The data represent mean ± S.E.M. of three experiments.

This allowed us to determine the length of time at 65°C needed to destroy intestinal alkaline phosphatase activity. We found, by heating extracts of HeLaS3 cells to 65°C for varying lengths of time, that approximately 60% of alkaline phosphatase activity in HeLaS3 cells is attributable to heat-stable PLAP activity and the remaining 40% of activity had the heat-lability (destroyed by 15 min at 65°C) characteristic of intestinal alkaline phosphatase activity. In the following experiments cell extracts were heated to 65°C for 20 min to ensure only PLAP activity was measured.

3.2. *Compounds which induce PLAP activity cause a decrease in cell proliferation*

We studied the effect of varying the concentration of butyrate, dexamethasone (a synthetic glucocorticoid), bromodeoxyuridine and dibutyryl cAMP on PLAP activity and cell growth. We also measured cell viability of HeLaS3 cells grown in the maximal concentrations of these compounds. The viability of the cells varied between 89–97%. Therefore the observed reductions in cell number were not the consequence of a decreased cell viability.

Figs. 1–4 show the results of these experiments. From these figures one can see that in each case there is an inverse correlation between PLAP activity and cell number (and therefore in growth rate). In each case the graphs of cell number against agent concentration form an almost perfect mirror image of the PLAP activity versus agent concentration graphs. We applied statistical analysis to these data by plotting activity versus 1/cell number on a scatter plot and found the correlation coefficients of these graphs varied between 0.680 and 0.951, indicating a high degree of correlation between PLAP activity and cell growth rate.

3.3. *Mimosine a compound which arrests the cell cycle in G1 induces PLAP activity*

Butyrate and prednisolone have previously been shown to block the cell cycle in G1 phase [11,13]. Since we had data indicating that these compounds also induce PLAP activity we decided to test whether another compound known to arrest the cell cycle in G1 would cause a corresponding induction in PLAP activity. We used the compound mimosine (α-amino-β-(3-hydroxy-4-oxo-1,4,dihydropyridine-1-yl)propanoic acid) which is a plant amino acid that inhibits cell cycle traverse in late G1, prior to the onset of DNA synthesis [14,15]. The results of this experiment are shown in Table I and demonstrate that mimosine totally inhibits growth of HeLaS3 cells while only causing a slight decrease in cell viability. At the same time this compound caused an increase in PLAP activity of up to 230%.

Figure 3 Effect of concentration of bromodeoxyuridine on placental alkaline phosphatase activity and cell growth rate of HeLaS3 cells.

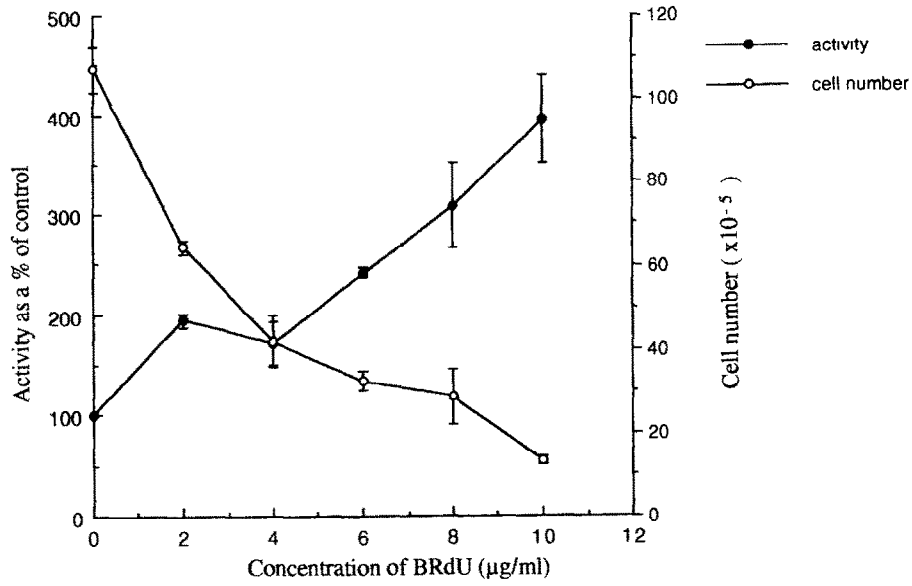

Fig. 3. HeLaS3 cells were seeded into 75 cm^2 flasks at approx. 8×10^5 cells/flask and allowed to attach over a 24 h period. They were then grown, either in the absence (control) or presence of bromodeoxyuridine, at the indicated concentrations, for 6 days. Cells were then harvested for cell counting and for assay of heat-stable alkaline phosphatase activity. The medium was changed every 24 h. The data represent mean ± S.E.M. of three experiments.

3.4. *EGF increases growth rate of HeLaS3 cells and causes a cell density-dependent decrease in PLAP activity*

We next investigated whether the inverse correlation between cell growth rate and PLAP activity would be maintained when the growth of HeLaS3 cells was stimulated by a growth factor. To determine this we grew HeLaS3 cells in serum free medium in the absence and presence of EGF. We measured the increase in cell number at various times after the addition of EGF and at the same time measured PLAP activity.

The growth response of HeLaS3 cells to EGF is shown in Fig. 5a. This graph shows that EGF at a concentration of 20 ng/ml significantly increases the growth rate of HeLaS3 cells. Cells still grew in the absence of EGF, but at a considerably slower rate than in its presence. Fig. 5b shows the corresponding PLAP activities of the same cells used in the experiments represented in Fig. 5a. This shows that after an initial 24 h of growth in the presence of EGF there was no measurable decrease in PLAP activity, but between the 2nd and 4th days of culture there was a small, measurable decrease in PLAP activity. We wondered whether the fact that there was no decrease in PLAP activity over the initial 24 h period in the presence of EGF might be due to the lower cell density of these cells, that is whether the decrease in activity observed in the presence of EGF was cell density-dependent. To test this idea we repeated the experiment, seeding the cells at the higher density of 3×10^6 cells/flask. The experiment was performed in triplicate and gave the following results: control activity (at 24 h) – 0.0047 ± 0.0004 units/mg and +EGF (at 24 h) – 0.0037 ± 0.0002 units/mg. These results suggest

Table I

Effects of mimosine on cell growth and placental alkaline phosphatase activity of HeLaS3 cells

Culture time (h)	Cell number		PLAP activity (% control)	
	control	+mimosine	control	+minosine
24	94.7 ± 2.4	43.0 ± 2.2	100 ± 2	175 ± 7.5
48	131 ± 14.9	44.3 ± 2.3	150 ± 17.5	275 ± 25
72	140 ± 14.2	44.5 ± 3.2	325 ± 25	750 ± 150

HeLaS3 cells were seeded at 44×10^5 cells/75 cm^2 flask and incubated for 12 h prior to growth in the absence (control) or presence of 400 μM mimosine, for the indicated times. Cells were harvested for counting, viability measurement and assay of PLAP activity. Results are presented as cell number ($\times 10^{-5}$) and as % of control enzyme activity (where control activity at 24 h is taken to be 100%) ± S.E.M. of three experiments. Viability of cells grown in the presence of mimosine was 92% at 24 h, 89% at 48 h and 88% at 72 h.

Figure 4 Effect of concentration of dibutyryl cAMP on placental alkaline phosphatase activity and cell growth rate of HeLaS3 cells.

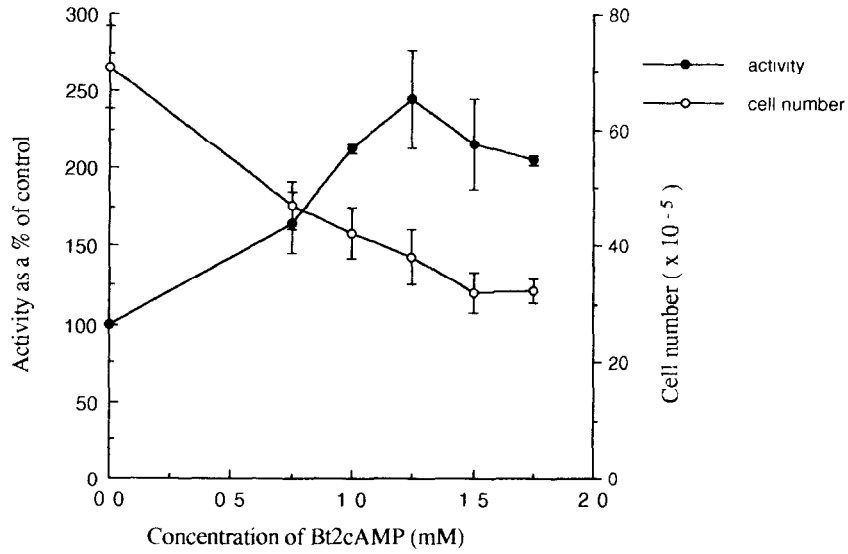

Fig. 4. HeLaS3 cells were sseeded into 75 cm^2 flasks at approx. 8×10^5 cells/flask and allowed to attach over a 24 h period. They were then grown, either in the absence (control) or presence of dibutyryl cAMP, at the indicated concentrations, for 72 h. Cells were then harvested for cell counting and for assay of heat-stable alkaline phosphatase activity. The medium was changed every 24 h. The data represent mean ± S E.M of three experiments.

that the reduction of PLAP activity observed when HeLaS3 cells are grown in the presence of EGF is indeed cell density-dependent.

4. DISCUSSION

We have presented data which show that there is an inverse correlation between cell growth rate and PLAP activity in HeLaS3 cervical cancer cells. We base this conclusion on three lines of evidence, firstly we have shown that four compounds that induce phosphatase activity cause a coincidental, dose-dependent, decrease in growth rate, secondly we have shown that mimosine, a compound which prevents cell growth by arresting the cell cycle in G1 phase, simultaneously increases PLAP activity and thirdly we have shown that increasing the growth rate of HeLaS3 cells, by culturing them in the presence of EGF, causes a concomitant decrease in PLAP activity. This decrease in PLAP activity seemed to be cell density-dependent.

As yet this is simply a correlation and it is not possible to say exactly how these two variables are connected. Thus, we cannot say whether it is the increase in PLAP activity which somehow brings about a decrease in growth rate, or whether the reverse is true, that is whether a decrease in growth rate in some way triggers an increase in PLAP activity. It is tempting to see significance in the reported protein phosphotyrosyl phosphatase activity of alkaline phosphatases [6,8] since stimulation of cell growth rate by certain growth factors is recognized as being accompanied by an increase in protein phophotyrosine kinase activity [16]. The EGF receptor belongs to a class of hormone/growth factor receptors that possess tyrosine kinase activity [17]. The suggestion would therefore be that, if stimulation of cell division rate involves an intracellular signalling mechanism requiring increased tyrosine kinase activity, it would seem logical if decreased cell division involved increased protein tyrosine dephosphorylation [18].

On the other hand the increase in PLAP activity might be a consequence rather than a cause of decreased cell proliferation. It is a general observation, in both normal and neoplastic cellular systems, that there is a correlation between increased state of differentiation and decreased tendency to undergo cell division. Several of the agents that induce PLAP in cultured cells are also capable of inducing the differentiation of cells in vitro [19,20]. The induction of differentiation in vitro of normal human neutrophils and of murine B cells has been shown to be accompanied by increased alkaline phosphatase levels [21,22]. Cellular differentiation is the consequence of changes in the array of genes that are expressed in a cell. The induction of PLAP activity in HeLa cells has been shown to involve increases in PLAP

Figure 5 Effect of EGF on (a) cell growth rate and (b) placental alkaline phosphatase activity of HeLaS3 cells

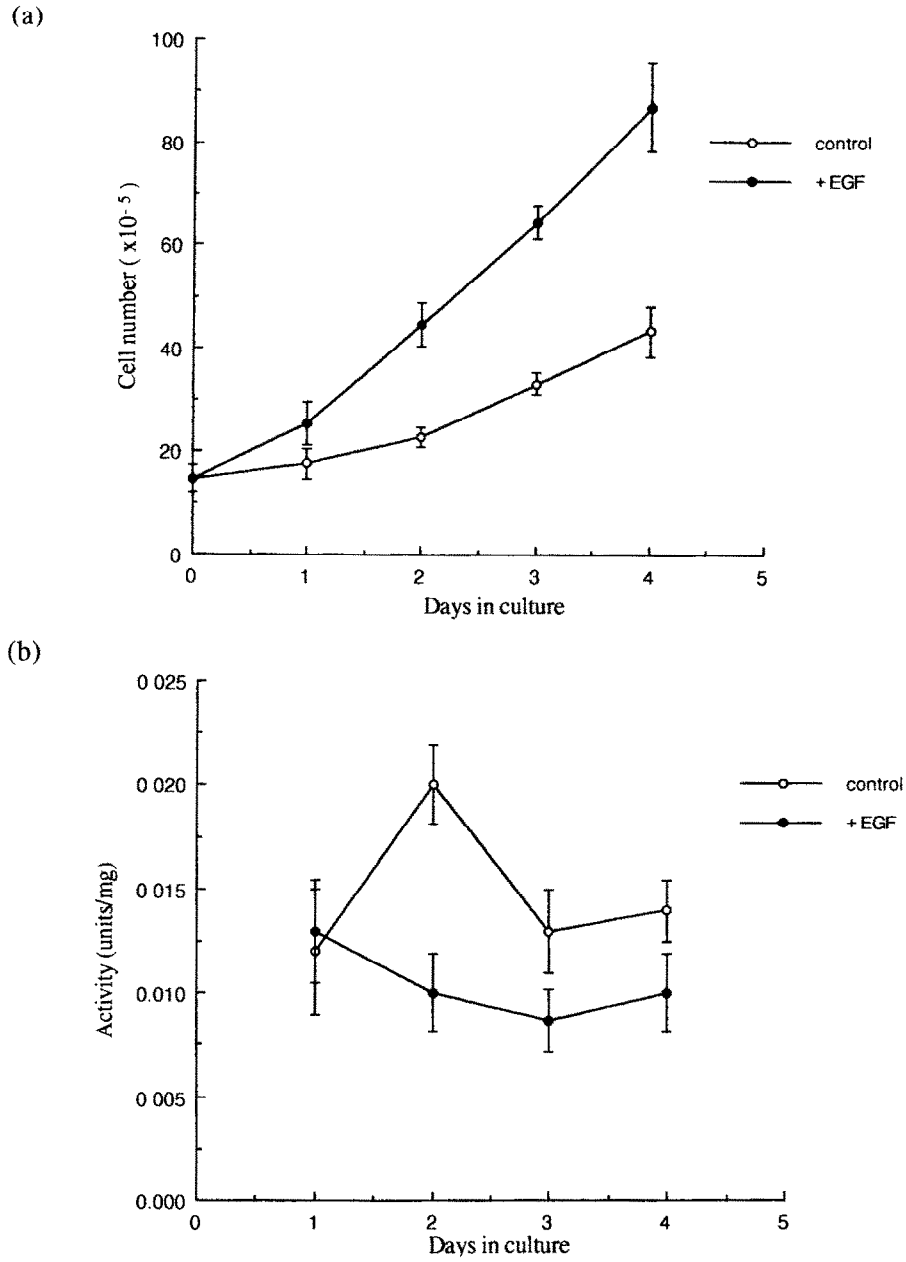

Fig. 5. HeLaS3 cells suspended in MEM containing 2.5% FCS were seeded into nine 75 cm^2 flasks to give approx. 16×10^5 cells/flask. Following a period of 24 h, to allow attachment, the cells were washed 3 times in PBS, before addition of serum-free medium. The cells were grown in serum free medium for 24 h after which time they were grown, either in the absence (control) or presence of 20 ng/ml EGF. Cells were harvested every 24 h (a) for cell counting and (b) for heat-stable alkaline phosphatase assay. The data represent mean ± S.E.M. of three experiments.

mRNA, presumably as a result of increased gene transcription [9,23].

Our findings therefore may be seen as establishing a clear link between expression of the placental alkaline phosphatase gene and rate of cell division. It is not as yet possible to be certain what is the nature of this connection but one possibility is that, by virtue of its protein tyrosine phosphatase activity, PLAP may be a crucial component of the growth regulatory mechanism.

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