Placental alkaline phosphatase isoenzyme expression by the non-HeLa DoT cervical-carcinoma cell line

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Expression of the oncodevelopmental protein, placental alkaline phosphatase, was observed in DoT cells, an epidermoid cell line derived from cervical carcinoma. Under normal conditions of growth *in vitro*, biochemical inhibition, cytochemical and immunological studies revealed that these cells express the term-placental (Regan) isoenzyme. Thus, alkaline phosphatase activity was observed to be heat-stable and inhibited by L-phenylalanine. These properties, supported by immunoelectrophoretic analysis using antisera specific for liver, intestinal or term-placental isoenzymes, identified the isoenzyme as placental type. DoT cells treated with prednisolone (1 µg/ml) increased total alkaline phosphatase specific activity. This activity was also identified as term-placental phosphatase isoenzyme. On the other hand, treatment of the same cells with sodium butyrate (1 mM) did not induce increased activity of the term-placental isoenzyme, an unexpected observation. As a result of these studies, DoT cells are proposed as a representative cell line for studies of the regulation of oncodevelopmental gene expression in human tumour cells of cervical origin.

The detection of the term-placental isoenzyme of alkaline phosphatase (Regan isoenzyme) as well as other placental or foetal gene products in a variety of different human cancers had led to the hypothesis that the derepression of developmental genes may be involved, either directly or indirectly, in the malignant process. Although the term-placental (Regan) isoenzyme has been shown to be expressed by a variety of different tumour types (Nathanson & Fishman, 1968; Jacoby & Bagshawe, 1971; Miki *et al.*, 1976), the highest incidence appears to be in malignancies of gynaecological origin (Stolbach *et al.*, 1976; Benham *et al.*, 1978). In this respect, it is not surprising that many of the cell lines derived from the original HeLa cervical adenocarcinoma have also been shown to express term-placental type alkaline phosphatase activity when grown *in vitro* (Elson & Cox, 1969; Bottomley *et al.*, 1969; Fishman & Singer, 1975).

A great deal of interest in examining mechanisms of gene regulation which result in expression of a foetal phenotype in neoplasia has resulted from the establishment of these continuous cell lines. Such studies have been aided by the identification of agents such as prednisolone (Cox & MacLeod, 1961; Herz, 1973) and sodium butyrate (Griffin

et al., 1974), which alter the degree of expression of certain of these foetal gene products *in vitro*.

Most of the cell lines that have been used as model systems in such studies are derivatives of a cervical adenocarcinoma cell line, HeLa, which have lost the original phenotypes and are thus not representative of the majority of the cervical carcinomas that are observed clinically. Pattillo (1977) reported establishment of the DoT cell line, which was derived from an epidermoid carcinoma of the cervix. This malignant cell type is observed in approx. 95% of all cases of cervical cancer. Further studies by these same workers indicate that the B-subunit of human chorionadotropin, which is expressed by these cells, can be induced to much higher amounts after treatment with sodium butyrate (Hussa *et al.*, 1978).

In the present paper, the expression and modulation of a second trophoblast product in this cell line is described. By biochemical and immunological criteria, it was demonstrated that the DoT cell line expresses an alkaline phosphatase isoenzyme that has properties identical with those of the isoenzyme from third-trimester placenta. Modulation of enzyme activity by prednisolone but not by sodium butyrate was detected. It is proposed that this cell line may serve as a more reliable model cell system for the study of the mechanism of developmental gene expression by this type of gynaecological tumour.

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Experimental

Cells and cultivation

The DoT cell line was a generous gift from Dr. R. A. Pattillo. This cell line originated from a case of epidermoid cervical carcinoma and represents the malignant cell type observed clinically in most cancers of the cervix (Pattillo, 1977). Cells were grown in 25 ml tissue-culture flasks at 37°C under 5% CO₂ in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum and antibiotics.

Preparation of extracts

The cells were harvested and washed twice with physiological saline by resuspension and centrifugation. The pellet, collected in a 7 ml plastic tube, was then suspended in 1 ml of 0.05 M-Tris/HCl, pH 8.6, and sonicated for 10 s at the lowest power setting of the Bronwill Biosonik IV. The specific activity of such extracts range between 13 and 15 nmol of *p*-nitrophenol released/min per mg of protein.

Alkaline phosphatase analysis

Total alkaline phosphatase activity of all extracts was determined by the method of Lowry (1957), with *p*-nitrophenyl phosphate as substrate. Inhibition of activity in presence of various concentrations of the organ-specific inhibitors L-phenylalanine or L-homoarginine was performed as described previously (Fishman *et al.*, 1971). Heat-stability of the enzyme was determined by assay of activity remaining after heating of samples for various times at 65°C (Fishman *et al.*, 1971). Disc-gel-electrophoretic separation of isoenzymes was carried out in 7.5% acrylamide gels by the method of L. Fishman (1974). Enzyme activity was detected in gels by the method of Sasaki & Fishman (1973), which utilizes α -naphthyl acid phosphate as substrate and coupling of the reaction product to aminodiphenylamine diazonium salt (L. Fishman, 1974).

Immunological identity of isoenzymes was established by preincubation of extracts separately with appropriate dilutions of antisera (Fishman *et al.*, 1976) directed toward purified liver, intestinal or term-placental isoenzymes before disc-gel electrophoresis. Retardation by antibody of the migration of enzyme activity during electrophoresis identifies the enzyme antigen as having the determinants recognized by the specific antiserum because the antigen-antibody complex will precipitate and remain near the origin. A positive test is the absence or retardation of the antigen band.

Cytochemical analysis

The two-stage technique of Sasaki & Fishman (1973) was employed. Initially, the washed formol/calcium-fixed smears on cover slips were preincu-

bated separately in ice for 2 h in the presence of 50 mM-L-phenylalanine, D-phenylalanine or L-homoarginine in 0.05 M-propanediol buffer at pH 9.6. In the next phase, the cover slips were washed and transferred to mixtures containing amino acid as before but in the presence of 44 mM-sodium α -naphthyl acid phosphate and 1 mg of Fast Blue BBN/ml. Incubation was done at room temperature for 30 min.

Appropriate control digests were included. Thus, the D-phenylalanine digests served as control for the L-phenylalanine inhibitor while the digest lacking amino acid was the control for the L-homoarginine inhibitor. In this way, one can identify cells which express either the term- or the early-placental isoenzyme of alkaline phosphatase.

Modulation of alkaline phosphatase activity

The effects of agents known to induce expression of the term-placental isoenzyme of alkaline phosphatase in certain HeLa cell lines were examined. Prednisolone (1 μ g/ml final concn.) or sodium butyrate (1 mM final concn.) was added to 72 h cultures of cells growing in RPMI 1640 medium (approx. 10⁵ cells/25 ml flask). At intervals after addition of modulator, cells were harvested. Both enzyme specific activity and isoenzyme profile were determined as described above and compared with those of untreated control cell cultures harvested at the same time.

Results and discussion

Enzyme inhibition tests

Examination of alkaline phosphatase expressed by the DoT cell line by several methods revealed that this enzyme had properties identical with those of the term-placental isoenzyme expressed in normal third-trimester placenta.

Fig. 1 demonstrates the results of experiments in which a comparison was made of the effects of various concentrations of the inhibitors, L-phenylalanine and L-homoarginine, and of various intervals of heat treatment at 65°C on enzyme extracts from both DoT cells and term placenta. Significant inhibition of the term-placental isoenzyme by mM concentrations of the L-isomer of phenylalanine has been reported by many workers and represents a commonly used test to distinguish this enzyme from the liver-type isoenzyme forms (W. H. Fishman, 1974). As shown in Fig. 1(a), the inhibitor profile of both enzymes over the range 0.01–10.0 mM-L-phenylalanine was identical.

In like fashion, the patterns of activity demonstrated in Fig. 1(b) represent the activity of enzymes in the presence of various concentrations of L-homoarginine, a specific inhibitor of the liver-type alkaline phosphatases. Neither enzyme was inhibited

Fig. 1. Comparative inhibition profiles of DoT-cell (○) and term-placental (●) alkaline phosphatases

Inhibition of activity was measured: (a) at various concentrations of L-phenylalanine, (b) at various concentrations of homoarginine, and (c) after various times of heating at 65°C.

significantly even at the highest concentration (10mM) of L-homoarginine. At this same concentration, alkaline phosphatases of the liver type are inhibited by 75–80% (W. H. Fishman, 1974).

Another test commonly used for characterization of term-placental isoenzyme is based on the resistance of the enzyme to heat inactivation at 65°C. All other alkaline phosphatases are rapidly inactivated at this temperature. As shown in Fig. 1, heat treatment of both DoT-cell enzyme and term-placental enzyme for up to 25 min at 65°C failed to cause significant loss of activity. In fact, it was our impression that the tumour-cell activity was more resistant to the effects of heat than was the term-placental isoenzyme itself. Since the tumour-cell preparations were sonicated preparations of whole cells, it is possible that existing associations of enzyme protein with membrane lipid components could account for the more heat-stable nature of the activity.

Cytochemical observations

Since the comparison of differential inhibition effects involved the use of sonicated whole cells, it was not possible to determine if the term-placental isoenzyme was expressed by all cells in the sample or by a sub-population. For this reason, it was necessary to use cytochemical staining of actively growing cell layers in the presence or absence of the differential amino acid inhibitors L-phenylalanine and homoarginine (Fig. 2).

All cells in the control cultures expressing activity could be detected by this method (Fig. 2). A small number of cells showed increased amounts of enzyme, as reflected by a greater accumulation of reaction product; however, such differences would be expected, since the cultures were not synchronous [Singer & Fishman (1976) have shown previously that cell-cycle variations occur with respect to expression of alkaline phosphatase.] When D-phenylalanine was included in both the preincubation mixture and the activity-staining assay mixture as a control, no inhibition was observed (Fig. 2b). If L-phenylalanine replaced the D-phenylalanine, complete inhibition of the activity stain was seen (Fig. 2c). Also, the inability of homoarginine to inhibit enzyme activity *in vitro* was confirmed as well (Fig. 2d). This result ruled out any significant expression of early placental or liver-type alkaline phosphatase isoenzyme by these cells.

Immunoelectrophoretic studies

When the antigenic characteristics of the alkaline phosphatase expressed by DoT cells were examined by immunoelectrophoretic techniques, the patterns illustrated in Fig. 3 were obtained. The first gel (Fig. 3a) shows migration of enzyme (in absence of monospecific antisera) in 7.5% polyacrylamide disc gels containing 0.1% Nonidet P40. The presence of detergent in both gels and samples was required in order to obtain migration of enzyme into the gel matrix; nevertheless, some enzyme remains at the origin.

The next three gels (Figs. 3b–3d) show the results when enzyme extracts had been incubated with antisera directed toward purified liver, intestinal or term-placental isoenzymes respectively before electrophoresis. Of the three antisera tested, only that specific for the term-placental isoenzyme reacted with the DoT-cell enzyme, as shown by the retardation of migration of the specific antigen antibody complex into the gel matrix. A band (Fig. 3d) did not move out into the gel. This immunological analysis confirmed the inhibitor and cytochemical results, which indicated that this cell line expressed only term-placental isoenzyme.

Effect of prednisolone and butyrate

Agents such as prednisolone and butyrate have

Fig. 2. Cytochemical characterization of DoT-cell alkaline phosphatase

Cells were stained: (a) in absence of specific inhibitor (control); (b) in the presence of 50 mM-D-phenylalanine; (c) in the presence of 50 mM-L-phenylalanine; (d) in the presence of 50 mM-homoarginine. The bar in (a) represents 100 μ m. Since no counterstain was employed, morphological detail in (c) is not visible.

Table 1. Time course of alkaline phosphatase activity of DoT cells in presence of modulating agents
The results are from a representative experiment. Abbreviation used: N.D., not determined.

Modulating agent in culture	Time (days)	Specific activity (nmol of <i>p</i> -nitrophenol formed/min per mg of protein)	Inhibition (%) by		
			L-Phenylalanine	L-Homoarginine	Heat treatment
None	2	21.8	100	40	4
Prednisolone	2	32.8	86	27	4
Butyrate	2	23.9	86	35	6
None	6	25.7	83	24	4
Prednisolone	6	72.3	72	35	1
Butyrate	6	17.1	63	38	4
None	11	56.4	73	31	4
Prednisolone	11	134.7	75	26	4
Butyrate	11	N.D.	N.D.	N.D.	N.D.

been shown to be modulators of oncodevelopmental gene expression in other cell lines. Their effects were examined in the DoT cells as a function of time after introduction of the agent into actively growing cultures (Table 1). Control cultures lacking the modulator agents did not show appreciable increases initially but, by day 11, specific activity was

approximately doubled. Hormone treatment of cells had little effect after 2 days but, by day 6, specific activity was 2.8-fold that of control cells, and this was maintained after extended growth for 11 days. On the other hand, treatment of cells with 1 mM-butyrate did not induce alkaline phosphatase activity. At day 6, enzyme specific activity was 33%

Fig. 3. Immunoelectrophoretic analysis of DoT-cell alkaline phosphatase in 7.5% polyacrylamide gels (a) Cell extract, no antiserum (control). (b) Cell extract plus anti-(liver alkaline phosphatase) serum. (c) Cell extract plus anti-(intestinal alkaline phosphatase) serum. (d) Cell extract plus anti-(term-placental alkaline phosphatase) serum. Some high-molecular-weight aggregates of alkaline phosphatase remain at the origin in all four lanes. Only in (d) is the DoT isoenzyme prevented from migrating by the placental-specific antibody.

gonadotropin by 67 different human tumour cell lines growing *in vitro*.

The cell lines adopted as the most convenient models for studies of gene regulation have been derived from HeLa cells, an adenocarcinoma of the cervix. However, as pointed out by Pattillo (1977), the DoT epidermoid cell line is more representative of the majority of cervical cancers observed clinically. In fact, 95% of all cervical carcinomas are derived from this cell type. In the present paper, we have demonstrated that the DoT cell line expresses an alkaline phosphatase activity which can be classified as the Regan or term-placental type isoenzyme and that known modulating agents affect the extent of enzyme expression in discordant fashion. In addition, it appears from this result and those of others (Hussa *et al.*, 1978) that the DoT cells exhibit differential modulation of expression of two onco-developmental gene products by the same agent. For these reasons, it is suggested that the DoT cell line may serve as a more representative model system available for studies of the mechanism(s) of regulation of these genes in tumours of gynaecological origin.

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less than that of the control cells and by day 11 many of the cells had detached from the culture flask and were non-viable.

Several authors have reported that many HeLa-cell lines and other established tumour-cell lines which express the term-placental-type isoenzyme can be induced to express higher activity in the presence of these same modulating agents (Cox & MacLeod, 1961; Herz, 1973; Griffin *et al.*, 1974). It is not clear why the DoT cell, which expresses the term-placental isoenzyme and which responds to prednisolone treatment, is not similarly induced by butyrate.

Other workers have reported that a second oncotrophoblast protein can be induced in DoT cells by 1 mM-butyrate (Hussa *et al.*, 1978). Such a finding is noteworthy when one considers the report by Neuwald *et al.* (1980), in which it was shown that, in most cases, expression was discordant of any two oncodevelopmental markers including term-placental alkaline phosphatase and human chorio-

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