Alkaline phosphatase protein increases in response to prednisolone in HeLa cells

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Quantification of term-placental alkaline phosphatase isoenzyme protein in HeLa TCRC-1 cells grown in the presence and absence of prednisolone indicates that there is a net increase in amount of enzyme-specific protein in prednisolone-stimulated cells. In a similar analysis of HeLa D98AH2 cells, prednisolone treatment causes the appearance of term-placental alkaline phosphatase protein and the loss of the intestinal isoenzyme protein. These results support the interpretation that the response of these cells to corticosteroids is the net accumulation of alkaline phosphatase protein rather than the modification of pre-existing enzyme to a more active state.

The production of alkaline phosphatase (EC 3.1.3.1) by HeLa cells (Elson & Cox, 1969; Fishman, 1969) has stimulated investigation into the control of modulation of this enzyme in cultured human cells. Thus modulation of enzyme activity by a variety of agents, including glucocorticoids (Cox & Macleod, 1961), hyperosmolarity (Nitowsky *et al.*, 1963), substrates (Cox & Pontecorvo, 1961; Griffin & Cox, 1966), butyrate (Griffin *et al.*, 1974), halogenated pyrimidines (Goz, 1974; Goz & Walker, 1976) and methotrexate (Speeg *et al.*, 1976), has provided a set of conditions that may prove useful in unravelling the control of alkaline phosphatase gene expression.

The cell-surface glycoprotein alkaline phosphatase has been shown to exist in a number of different isoenzymic forms named for the tissue in which they predominate, such as term placenta, intestine, bone, liver, and kidney (Fishman, 1974; Moss, 1977). The term-placental and intestinal isoenzymes each appear to be encoded by a distinctive gene locus, whereas the bone, liver and kidney forms share a single locus (Fishman, 1974; Moss, 1977; Mulivor *et al.*, 1978*a,b*). The isoenzymic forms of alkaline phosphatase are identifiable by their electrophoretic mobilities, inhibition by specific amino acids and peptides, thermal stabilities, immunological reactivities and peptide fingerprints (Fishman, 1974; Moss, 1977; Doellgast & Fishman, 1977; Fishman *et al.*, 1971).

The modulation of alkaline phosphatase activity in HeLa cells by prednisolone and butyrate is accompanied by enhancement or suppression of specific isoenzymic forms. HeLa TCRC-1 cells,

which express term-placental alkaline phosphatase (Singer & Fishman, 1974), exhibit increased term-placental alkaline phosphatase activity on growth in prednisolone or butyrate (Kottel & Fishman, 1978). HeLa D98AH2 cells normally express intestinal-type alkaline phosphatase and lose activity on growth in prednisolone (Kottel & Fishman, 1978). The components of this diminished activity include the appearance of placental alkaline phosphatase and a nearly complete absence of intestinal activity. On the other hand, both activity and isoenzyme profile remain relatively unchanged on growth of D98AH2 cells in the presence of butyrate.

Modulations of enzyme activity may be caused by either changes in amount of enzyme protein, alterations in the catalytic capacity of existing enzyme molecules or a combination of these two. Cox *et al.* (1975) report that the effect of hormone induction is a modification of HeLa-cell alkaline phosphatase rather than an increase in enzyme amount. This putative enzyme modification would result in alkaline phosphatase molecules with higher catalytic activity. They find that both RNA and protein synthesis are required for this activity increase, yet believe that alkaline phosphatase is not among the proteins synthesized. This view has been the exception to the rule that corticosteroids produce increases in enzyme protein. On the other hand, Singer & Fishman (1975) found no evidence for prednisolone-enhanced alkaline phosphatase with higher catalytic activity per unit of protein.

We present experimental results indicating that the prednisolone-stimulated increase in placental alkaline phosphatase in cell line HeLa TCRC-1 is

due to a corresponding increase in the quantity of alkaline phosphatase protein. Also, the shift in isoenzyme activity apparent in line D98AH2 on growth in prednisolone is shown to be reflected in a loss of intestinal-isoenzyme protein and the appearance of placental alkaline phosphatase protein.

Materials and methods

Cell culture

HeLa-cell lines were grown in 25 cm² plastic flasks in RPMI-1640 media (Irvine Scientific, Fountain Valley, CA 92708, U.S.A.) supplemented with 10% (v/v) foetal bovine serum and antibiotics. After inoculation at a density of 10⁵ cells/flask, the cultures were allowed to incubate at 37°C in an atmosphere containing 5% CO₂ for 1 day before receiving either prednisolone (final concn. 1 µg/ml) dissolved in 95% (v/v) ethanol or ethanol alone as a control. After a further 5 days of growth, the cells were harvested by trypsin treatment (Knaup *et al.*, 1978) and processed as described below.

Cell processing

After harvest, the cells in each culture were first counted and then each culture was split into two equal portions. One portion was sonicated and assayed for alkaline phosphatase activity as described by Kottel & Fishman (1978) using *p*-nitrophenyl phosphate (Sigma) as substrate, and for protein content by the method of Lowry *et al.* (1951) with bovine serum albumin (Nutritional Biochemicals) as protein standard. The other portion was incubated in bromelain to solubilize the surface-bound alkaline phosphatase as described by Kottel & Hanford (1980).

Rocket immunoelectrophoresis

After centrifugation to sediment the cells, the solubilized alkaline phosphatase activity was determined and the supernatant fluid containing the liberated alkaline phosphatase was analysed for amount of placental alkaline phosphatase specific protein by the method of rocket immunoelectrophoresis (Laurell, 1972). The rockets were made visible by staining for enzymic activity by using a reaction mixture consisting of 50 mg of Fast Blue salt R (Dajac Laboratories, Philadelphia, PA, U.S.A.), 50 mg of α -naphthyl acid phosphate (Sigma) and 10 mg of activated charcoal dissolved in 25 ml of 0.75 M-2-amino-2-methylpropan-1-ol, pH 10.15 (Baker). The relative amounts of alkaline phosphatase specific protein were determined by a comparison of the areas under the rockets and were related to the enzyme activity for both control and prednisolone-treated cells.

Antisera

Antisera used were prepared as described by Doellgast *et al.* (1977).

Results and discussion

Although the modulations of alkaline phosphatase activity in various HeLa cell lines, including TCRC-1 and D98AH2, in response to prednisolone have been previously characterized (Kottel & Fishman, 1978), the molecular details of such responses remain controversial (Cox *et al.*, 1975; Singer & Fishman, 1975). In order to satisfy ourselves that the corticosteroid modulations of alkaline phosphatase activity are reflections of gene expression, we have here initiated experiments designed to characterize definitively these modulations.

HeLa TCRC-1 cells grown in the presence of prednisolone (1 µg/ml) in this experiment produce a 1.5–2.5-fold increase in total activity (Table 1). When expressed in terms of specific activity, the increase is about 3-fold. It may be noted that, while the prednisolone-treated cells proliferate more slowly than control cells, the prednisolone-treated cells have more protein (Table 1) and are visibly larger than control cells. For a comparison of the catalytic capacity of the induced versus the uninduced alkaline phosphatase, a measure of the total alkaline phosphatase activity is compared with the amount of alkaline phosphatase specific protein as determined immunochemically by using the method of rocket immunoelectrophoresis.

Rocket immunoelectrophoretic analysis of extracts of bromelain-treated control and induced cells reveals that the increase in activity is matched by an increase in alkaline phosphatase specific protein (Fig. 1 and Table 2), as indicated by the increased area defined by the rocket formed by extracts from prednisolone-treated cells. Table 2 presents an analysis of the rockets shown in Fig. 1, which are derived from a representative experiment. The experimental results include enzyme activity and the corresponding rocket areas. Analysis of these results involves the correlation of changes of enzyme activity (resulting from induction) and changes in rocket area. The analysis is best explained if we let *A* and *C* represent the relative areas of the rockets for uninduced and induced cultures and *B* and *D* represent the enzyme activities for uninduced and induced cells. The values to compare then are the ratios of *A/B* and *C/D*. For each experiment a similarity of *A/B* and *C/D* indicate that an increase in alkaline phosphatase protein accounts for the increased alkaline phosphatase activity. An analysis of the ratio of ratios [or (*A/B*)/(*C/D*)] from eight experiments yields a mean value of 0.94 ± 0.10 . The

Table 1. Summary of alkaline phosphatase activity changes in TCRC-1 cells in response to prednisolone

Cells were grown, harvested and assayed for alkaline phosphatase activity and protein as described in the Materials and methods section. Enzymic activity is expressed as nmol of *p*-nitrophenol formed from *p*-nitrophenyl phosphate/min per ml.

Growth conditions	Cell count on day 5	Protein concn. in sonicated cell portion (mg/ml)	Alkaline phosphatase activity in sonicated cell portion	Specific activity (units/mg of protein)
Control	102.5×10^4	1.25	46.2	36.6
+Prednisolone	26×10^4	0.81	76.0	93.8

Table 2. Analysis of rocket immunoelectrophoresis of alkaline phosphatase from HeLa TCRC-1 cells grown in the presence and absence of prednisolone

Cells were grown, harvested, processed and analysed as described in the legend to Fig. 1 and in the Materials and methods section. The rocket areas represent a relative value, with the area of the 'C' rocket assigned a value of 1.00. The areas were determined by tracing the outlines of the rockets on to paper, cutting out the paper tracings and weighing them.

Prednisolone	Enzyme activity applied (unit)	Relative area under rocket
Present (P)	0.60	1.86
Absent (C)	0.37	1.00
Ratio P/C	1.62	1.86

illustrated by the nature of the rockets in Fig. 2. Since the antiserum incorporated into the agarose matrix is anti-(placental alkaline phosphatase), the extract of cells grown in the presence of prednisolone results in a rocket on immunoelectrophoresis. However, the control culture extract formed only streaks indicative of alkaline phosphatase activity which does not cross-react with the anti-(placental alkaline phosphatase) serum. These results thus indicate that a non-cross-reactive form of alkaline phosphatase is produced by the control D98AH2 cell cultures, and on stimulation by prednisolone the cells shift from expression of this non-cross-reacting form to production of term-placental alkaline phosphatase.

Histochemical observations indicate that this shift in isoenzyme expression is not due to the selective stimulation by prednisolone of a genetically distinct sub-population of cells which normally express no alkaline phosphatase but which responds to prednisolone by making term-placental alkaline phosphatase. Parallel sparsely inoculated cultures of D98AH2 cells allowed to proliferate form distinct colonies with about 30 cells each were cultured with or without prednisolone for 10 days while maintaining their discrete colonies. Histochemical staining for alkaline phosphatase activity (Sasaki & Fishman, 1973), using the criteria of heat stability

Fig. 1. Rocket immunoelectrophoresis of TCRC-1-cell alkaline phosphatase

HeLa TCRC-1 cells were grown in the presence (P) and absence (C) of prednisolone. The cells were harvested, counted and processed as described in the Materials and methods section. A sample (8 μ l) of the appropriate bromelain supernatant fluid was loaded into each well in the agarose layer, into which had been incorporated anti-(term-placental alkaline phosphatase) sera. Electrophoresis was carried out at a current of 3mA/cm of gel. Staining was performed as described in the Materials and methods section.

simplest explanation is that the catalytic capacity of the alkaline phosphatase molecules in both cases is identical and that the difference in activity is due to a corresponding difference in amount of alkaline phosphatase protein.

HeLa D98AH2 cells, which produce intestinal alkaline phosphatase when grown in RPMI media with 10% foetal bovine serum, have been shown to express the term-placental isoenzyme on growth in prednisolone. This shift in isoenzyme expression is

Fig. 2. Rocket immunoelectrophoresis of D98AH2-cell alkaline phosphatase

HeLa D98AH2 cells were grown and processed as described for the HeLa TCRC-1 cells in the legend to Fig. 1.

(65°C for 5 min) and phosphate sensitivity (25 mM- Na_2HPO_4) for term-placental isoenzyme and heat lability and phosphate insensitivity for the intestinal isoenzyme, revealed that nearly all colonies cultured without prednisolone had intestinal-type activity, while the great majority of colonies exposed to prednisolone expressed the term-placental isoenzyme. Since the colonies were first established in the absence of prednisolone, and they continued to remain discrete during all subsequent incubations, the shift in isoenzyme expression by D98AH2 cells cannot be ascribed to a genetic sub-population of cells which have overgrown the whole culture, but must be a result of a change in the expression of a single cell-derived population from intestinal alkaline phosphatase to term-placental alkaline phosphatase.

In both of these cell lines, the result of prednisolone stimulation is an increase in term-placental alkaline phosphatase activity. Therefore, in view of the experimental results herein described this enhanced activity can be attributed to an increase in term-placental alkaline phosphatase protein, indicating that a modulation of alkaline phosphatase gene expression has taken place.

Since the amount of a given protein under steady-state conditions represents a balance between rates of enzyme synthesis and degradation, the alterations in quantity of alkaline phosphatase protein may be brought about by changes in either or both of these rates. Investigations of the rates of alkaline phosphatase synthesis and degradation in these and other

cell lines are required for further analysis and characterization of the modulation of alkaline phosphatase gene expression.

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References

- Cox, R. P. & Macleod, C. M. (1961) *Nature (London)* **190**, 85–87
- Cox, R. P. & Pontecorvo, G. (1961) *Proc. Natl. Acad. Sci. U.S.A.* **47**, 839–845
- Cox, R. P., Ghosh, N. K., Buzzell, K. & Griffin, M. J. (1975) in *Isozymes, I. Molecular Structure* (Markert, C. L., ed.), pp. 343–366, Academic Press, New York
- Doellgast, G. J. & Fishman, W. H. (1977) *Clin. Chim. Acta* **75**, 449–454
- Doellgast, G. J., Spiegel, J., Guenther, R. A. & Fishman, W. H. (1977) *Biochim. Biophys. Acta* **484**, 59–78
- Elson, N. A. & Cox, R. P. (1969) *Biochem. Genet.* **3**, 549–561
- Fishman, L., Inglis, N. R. & Fishman, W. H. (1971) *Clin. Chim. Acta* **34**, 393–400
- Fishman, W. H. (1969) *Ann. N.Y. Acad. Sci.* **166**, 745–759
- Fishman, W. H. (1974) *Am. J. Med.* **56**, 617–690
- Goz, B. (1974) *Cancer Res.* **34**, 2393–2398
- Goz, B. & Walker, K. P. (1976) *Cancer Res.* **36**, 4480–4485
- Griffin, M. J. & Cox, R. P. (1966) *J. Cell Biol.* **29**, 1–9
- Griffin, M. J., Price, G. H., Bazzell, K. L., Cox, R. P. & Ghosh, N. K. (1974) *Arch. Biochem. Biophys.* **164**, 619–623
- Knaup, G., Pfeleiderer, G. & Bayreuther, K. (1978) *Clin. Chim. Acta* **88**, 375–383
- Kottel, R. H. & Fishman, W. H. (1978) *Scand. J. Immunol. Suppl.* **8**, 571–574
- Kottel, R. H. & Hanford, W. C. (1980) *J. Biochem. Biophys. Methods* **2**, 325–330
- Laurell, C. B. (1972) *Scand. J. Clin. Lab. Invest.* **29**, (Suppl.) **124**, 21–37
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Moss, D. W. (1977) *Mol. Aspects Med.* **1**, 477–551
- Mulivor, R. A., Plotkin, L. I. & Harris, H. (1978a) *Ann. Human Genet.* **42**, 1–13
- Mulivor, R. A., Hannig, V. L. & Harris, H. (1978b) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3909–3912
- Nitowsky, N. M., Herz, F. & Geller, S. (1963) *Biochem. Biophys. Res. Commun.* **12**, 293–299
- Sasaki, M. & Fishman, W. H. (1973) *Cancer Res.* **33**, 3008–3018
- Singer, R. M. & Fishman, W. H. (1974) *J. Cell Biol.* **60**, 777–780
- Singer, R. M. & Fishman, W. H. (1975) in *Isozymes, 3. Developmental Biology* (Markert, C. L., ed.), pp. 753–774, Academic Press, New York
- Speeg, K. V., Jr., Azizkhan, J. C. & Stromberg, K. (1976) *Cancer Res.* **36**, 4570–4576