EFFECT OF PREDNISOLONE AND CONTACT PHENOMENA ON THE ALKALINE PHOSPHATASE ACTIVITY OF HEp-2 CELLS

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The role of prednisolone in the regulation of alkaline phosphatase of human cell cultures is ill defined. Several possibilities exist including: induction (Cox and MacLeod, 1961, 1962), repression (Erwin and Whitehead, 1964), altered protein conformation (Griffin and Cox, 1966), and effects on cell membranes (Melnykovych, 1966). The type of response observed is largely dependent on cell type, morphology, and nutritional factors (Cox and MacLeod, 1964). In an effort to resolve the role of prednisolone we have compared its effects on HEp-2 cells in stoppered-culture and perfused-culture systems. The latter system afforded control of nutritional factors and enabled us to assess effects of contact phenomena on the regulation of this enzyme by comparing its activity in pre- and post-confluent cell populations. As a result of these studies we wish to report that prednisolone has no effect on alkaline phosphatase activity per se but merely inhibits proliferation and increases the protein content of HEp-2 cells. In contrast, under perfusion conditions alkaline phosphatase activity is regulated by inherent control mechanisms of the cell which are sensitive to contact phenomena and population density changes. An analogy to this type of control observed in perfused

culture is made with that observed during in vivo processes of wound healing, malignant growth, and invasion of cells into subcutaneous sponge implants (Fell and Danielli, 1943; Biesele and Biesele, 1944; Woessner and Boucek, 1961).

MATERIALS AND METHODS

Human carcinoma (HEp-2) cells were obtained from the American Type Culture Collection, Rockville, Md., and grown in Medium 7a (cf. Kruse, Schoolar, and White, 1960) plus 10% whole calf serum. The perfusion technique employed was described previously (Kruse and Miedema, 1965). Periodic examinations of stock cultures for bacteria and mycoplasma were negative. Alkaline phosphatase activity of hypotonically-treated cells was measured colorimetrically (Melnykovych, 1962) by the release of p-NO_2-phenol in glycine buffer (0.12 M) pH 9.8 containing magnesium ions (1 mM) and at 25° C. Enzyme and substrate blanks were run simultaneously. Activity is expressed as units (one unit equals 0.001 Δ O.D. per hour) per 10^6 cells or per mg cell protein. Initial (zero hour) specific activity of HEp-2 cells for all perfusion experiments was 4363 $\pm$ 1361 units per 10^6 cells. Protein was measured by a modified Lowry procedure (Oyama and Eagle, 1956) and expressed as bovine serum albumin equivalents. Prednisolone (U.S.P.) was obtained from Calbiochem.

RESULTS AND DISCUSSION

The effects of prednisolone on stoppered cultures of HEp-2 cells for 48 hours are summarized in Table I. A linear log dose inhibition of proliferation occurred (26-60%), the protein content of the cells increased (110-130%), and so did the alkaline phosphatase activity (60-210%). These preliminary data imply that prednisolone had induced alkaline phosphatase activity. However, because of limitations in the mode of culture, it was not possible to

TABLE I

Effect of Prednisolone on Stoppered Cultures of HEp-2 Cells

Conc. of Prednisolone (μg/ml)	Proliferation Inhibition[a] (%)	Protein Content[b] (μg/10^6 cells)	Units Alkaline Phosphatase Activity[c]	
			10^6 cells	mg protein
0.00	0	210	1113	2547
0.25	26	440	1775	5785
2.50	46	468	3438	8698
25.00	60	480	2658	6142

[a]Hemocytometer counts of triplicate cultures at each concentration of prednisolone.

[b]Expressed as bovine serum albumin equivalents. Content of control cells at 0 hr was 185 μg/10^6 cells.

[c]Measured as the release of p-NO_2-phenol at 420 mμ in glycine buffer (0.12 M) pH 9.8. Activity at 0 hr was 1907 units/10^6 cells and 4728 units/mg protein; 1 unit is equal to 0.001 Δ O.D./hr.

rule out alternate possibilities for the increase in enzyme activity which include: (a) the effect of used medium and loss of cystine from fresh medium (Cox and MacLeod, 1964); (b) population density effects (Krooth, 1964; DeMars, 1964; Cox and MacLeod, 1962), and (c) phase of cell growth (Eker, 1965). Further studies on stoppered cultures were therefore abandoned in favor of perfused cultures (Kruse and Miedema, 1965) which circumvented most of the limitations inherent in the former method.

Here pH was controlled, nutrient levels were held essentially constant, and accumulation of metabolic products prevented. The concentrations of any added, or endogenous, activators or repressors were also maintained at

a constant level. Further, the enzyme activity in each experiment could be assessed in a "cell" culture phase, a "tissue" culture phase, and in cell populations proliferating at widely differing rates. Fig. 1 depicts the results of two such control experiments. In both cases, after a brief 1 to 2-day lag,

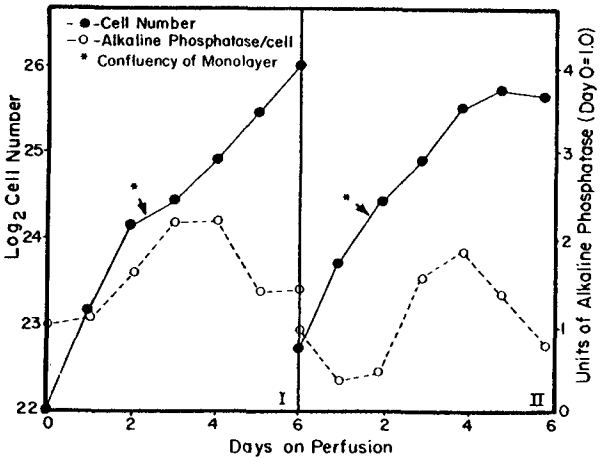

Fig. 1. - Alkaline phosphatase activity of HEp-2 cells cultured under perfusion conditions and its variation with population density. For method of determining enzyme activity see Methods section. Arrows denote confluency in culturing vessels.

enzyme activity increased (2- to 5-fold) during proliferation in the "cell" culture phase of the experiments (prior to confluency of the culturing vessel from days 0 to 3). Subsequently in the "tissue" culture phase of these experiments (after confluency and during formation of multilayers from days 4 to 6), the enzyme activity declined again to nearly day 0 levels. Cell protein content was essentially constant under these conditions as reported previously (Miedema and Kruse, 1965) and enzyme activity per mg of protein followed the same pattern as that on a per cell basis.

Supplementing the medium with prednisolone (25 μg/ml) reduced cell proliferation about 50% during a 6-day experiment (Fig. 2). Nevertheless, alkaline phosphatase activity increased to the same degree (2.3-fold) as control cultures during the "cell" culture phase of the experiment (on a per cell basis)

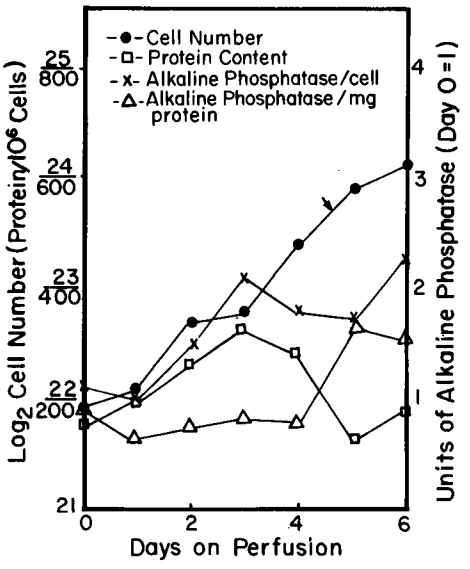

Fig. 2. - Effect of prednisolone (25 μg/ml) on alkaline phosphatase activity and protein content of HEp-2 cells. Protein content is expressed as microgram equivalents of bovine serum albumin per 1, 000, 000 cells. Arrows denote confluency in culturing vessels.

and remained high in the "tissue" culture phase. Protein content of cells treated with prednisolone exhibited a pattern similar to the enzyme activity (per cell) in the "cell" culture phase. Enzyme activity (per mg protein), however, remained constant during the "cell" culture phase while the protein content (per cell) increased but then enzyme activity rose sharply in the "tissue" culture phase when the protein content of the cells declined. It appeared that prednisolone had prevented the decline of enzyme activity in the "tissue" culture phase of the experiment. However, since proliferation was also inhibited about 50%, another experiment was conducted for 17 days (Fig. 3). In this experiment the alkaline phosphatase activity declined after day 10. This suggested that the decline in enzyme activity in the "tissue" phase was delayed in a manner consistent with inhibition of proliferation.

Thus, results from prednisolone-treated cultures substantiated control experiments, indicating variations in alkaline phosphatase activity were

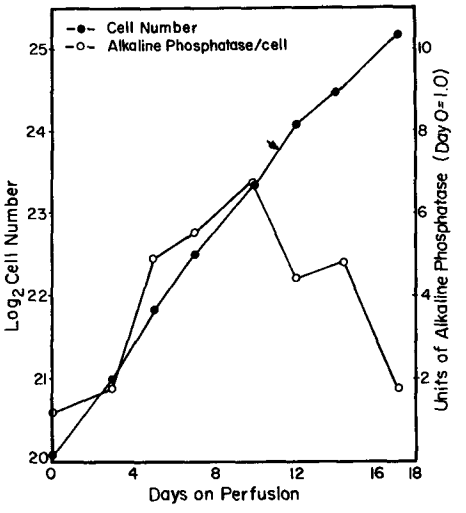

Fig. 3. - Effect of prolonged exposure of HEp-2 cells to prednisolone (25 μg/ml) on alkaline phosphatase activity and proliferation. Arrows denote confluency in culturing vessels.

related to the physiological disposition of the cells, i.e., whether they were in "cell" or "tissue" culture densities.

The use of perfusion cultures in lieu of stoppered cultures revealed an interesting facet concerning cellular regulation of enzyme activity. The growth of "cell" cultures into "tissue" cultures represents an in vitro model of hyperplasia. The variation in alkaline phosphatase activity observed here is very similar to changes occurring during the invasion of subcutaneous sponge implants by connective tissue cells (Woessner and Boucek, 1961). Woessner and Boucek have interpreted their observations as reflecting a correlation of enzyme activity to proliferation. A more plausible relationship, however, exists between high alkaline phosphatase activity and motility of cells in "cell" culture and between cessation of motility in "tissue" culture with a decline in enzyme activity despite continued proliferation. The significance of this observation to the problem of invasiveness by malignant cells

is suggested from histochemical studies on skin carcinomas (Biesele and Biesele, 1944) and from the correlation between alkaline phosphatase activity of L cells and their tendency to form nodules in vivo (Keefe, Merchant, and Kelsey, 1965). The inability of prednisolone to induce alkaline phosphatase activity in perfused cultures may reflect a prolonged turnover rate of the enzyme (Berlin and Schimke, 1965) or repression of its genome in cells undergoing division (Seidman, Teebor, and Becker, 1966). The delay in the decline of alkaline phosphatase caused by prednisolone is thought to reflect a stabilization of the enzyme (Griffin and Cox, 1966) or the cell membrane (Melnykovych, 1966) with which it is associated. The anomalous effect of prednisolone on the enzyme in stoppered cultures is presumably an artifact and merely reflects the inadequacy of the system for such studies.

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