Reversible Suppression of Alkaline Phosphatase in Human Thyroid Medullary Carcinoma Cells Transformed by SV40 (36202)

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Medullary carcinoma of the thyroid gland is a histologically distinctive neoplasm which most probably derives from calcitonin-secreting parafollicular cells or their embryologic predecessors of ultimobranchial origin (1). These tumors have been of considerable interest to endocrinologists and oncologists since they are hormonally active (2), and the susceptibility to neoplasia may be inherited as an autosomal dominant trait (3).

Tissue cultures established from medullary carcinomas of the thyroid gland may continue to produce calcitonin *in vitro* (4, 5), but the cells grow slowly and cannot be maintained for long periods. The technique of viral transformation with simian virus 40 (SV40) has been utilized to accelerate the growth rate and prolong the *in vitro* life span of such endocrine cells (4, 6).

Materials and Methods. Two thyroid carcinomas were found in a kindred with multiple endocrine neoplasia. One tumor was resected from a 56-year-old male (N. W.) and one from a 26-year-old male, a nephew of N. W. Calcitonin and typical secretory granules were in both tumors and an amyloid stroma was identified by Congo red staining and green birefringence.

Primary cultures were initiated with explants from fresh surgical specimens according to techniques previously detailed (4). Confluent cell sheets appeared within 21–30 days. At this time some of the primary culture flasks were inoculated with $10^{7.8}$ $TCID_{50}$ (tissue culture infective dose, 50% effective) of SV40. Approximately 30 days after infection, when the cells were in the first subculture, they showed the growth characteristics previously described in transformed human cell lines (6). As the monolayers from both control and infected flasks became confluent, they were separated from the flask surface with 0.25% trypsin, centrifuged at 150*g*, rinsed twice and dispersed in fresh medium (RPMI 1640 with 20% calf serum). Cultures were incubated at 37° and fed on alternate days. For alkaline phosphatase and protein determinations cultures were started from an original inoculum of 10^5 cells. At selected intervals the enzyme activity and protein were assayed as described previously (7).

Results. Alkaline phosphatase activity was demonstrated in cells from serial subcultures of the two uninfected thyroid carcinoma cell lines. The range of activity was from 0.2–0.6 units (units of activity are expressed as μmol of *p*-nitrophenol liberated in 30 min/mg of cell protein). Following transformation by SV40, the cells were carried through one additional subculture; at this point, about 30 days after infection, nearly all cells contained T antigen as demonstrated by immunofluorescence, and the enzyme assay revealed very depressed or undetectable levels of alkaline phosphatase. The range of activity was from 0.00–0.02 units/mg of cell protein, a maximum of 10% of the values seen in uninfected, nontransformed cultures.

Since alkaline phosphatase activity can be induced by adrenal corticosteroids in human leukocytes (8), chicken or mouse intestinal epithelium (9), and in certain tissue culture lines of human epithelial cells (10), the effect of hydroxycortisone acetate on the virus-transformed medullary carcinoma cells was examined. In these experiments steroid was added at a concentration of 2.5 μg/ml of culture fluid and the medium was replaced once every 48 hr throughout the experimental procedure. Representative results of the hydro-

TABLE I. Effect of Hydrocortisone Treatment on Alkaline Phosphatase in SV40 Transformed Medullary Thyroid Carcinoma Cells.

Days after adding steroid to media	Alkaline phosphatase[a]		
	Untreated	Line 1	Line 2
2	0.01	0.01	0.02
4	0.01	0.05	0.20
6	0.01	0.20	0.30
8	0.01	0.40	0.60

[a] The units of activity are expressed as micromoles of *p*-nitrophenol liberated in 30 min/mg of cell protein.

cortisone treatment in the two series of transformed tumor cells are shown in Table I. Over a treatment period of 10 days the amount of alkaline phosphatase activity consistently returned to preinfection levels. After 8–10 days the alkaline phosphatase levels did not show any further increase in the presence of hydrocortisone and by 12–14 days the continuous growth of cells produced such heavy monolayers that detachment from the surface of the flask usually occurred.

We did not demonstrate any steroid induction of alkaline phosphatase activity in uninfected thyroid carcinoma cells. Since the uninfected cultures survived for approximately 6 weeks, as opposed to about 7 months for the infected ones, esssentially all of the experiments were done after the demise of the uninfected cells.

Previous studies of HeLa S_3 cells have indicated that hydrocortisone diffuses back from the cell into the medium when the cells are incubated in fresh medium containing no hydrocortisone (10). Under these conditions alkaline phosphatase induction is completely reversible over a period of 72 hr. The thyroid carcinoma cells were tested to determine if the increase in enzyme activity was steroid dependent. Results of a representative experiment are shown in Table II and indicate that enzyme activity fell to pretreatment levels within 96 hr.

TABLE II. Effect of Hydrocortisone Withdrawal on Alkaline Phosphatase Levels in SV40 Transformed Medullary Thyroid Carcinoma Cells.

Samples tested	Alkaline phosphatase	
	Untreated	Line 1
No steroid		0.01
6 days of steroid treatment	0.01	0.07
8 days of steroid treatment	0.01	0.08
10 days of steroid treatment	0.01	0.30
[a]2 days after steroid withdrawal	0.01	0.05
[a]4 days after steroid withdrawal	0.01	0.01

[a] Hydrocortisone was withdrawn after 10 days of treatment.

When human cells are infected with SV40, it is usually possible to demonstrate infectious virus after transformation (11). Apparently a small fraction of human cells persist in the lytic phase. Titers of 10^4 $TCID_{50}$ of SV40 could be recovered from the serially passaged infected cultures of medullary carcinoma cells even though the majority of cells demonstrated morphological and functional evidence of transformation, with T antigen being present in nearly all cells. The titer remained the same in steroid-treated cultures, which excluded the possibility that hydrocortisone might enhance or suppress lytic infection.

TABLE III. Effect of SV40 Infection of African Green Monkey Kidney Cells on Alkaline Phosphatase Levels.

SV40 $TCID_{50}$	Maximum alkaline phosphatase activity (0–152 hr post infection)[a]
Uninfected	0.20
10^5	0.08
10^4	0.08
10^3	0.07

[a] At the end of 152 hr all of the cultures tested showed approximately 100% cytopathic effect.

Since there was production of SV40 in the system (10^4 $TCID_{50}$), we wished to determine if the formation of vegetative virus might contribute directly to any increase in enzyme levels. For this purpose a controlled infection of permissive African green monkey kidney (AGMK) cells was carried out. AGMK cells were infected with several $\log_{10}$ dilutions of stock SV40 containing 10^7 $TCID_{50}$, and daily enzyme assays were performed over a 6-day period. The results (Table III) showed a

definite decrease in alkaline phosphatase activity. Although this may represent only one example of a general depression of enzymatic activities associated with lytic infection, the experiment indicated that the production of SV40 was not associated with an increase in alkaline phosphatase activity.

Discussion. During early stages of chicken and mouse embryogenesis, alkaline phosphatase activity may be detected in all cells. As differentiation proceeds, the activity becomes confined to specific tissues and organs. During thyroid development in the rat, alkaline phosphatase localizes specifically in C cells derived from the ultimobranchial body (12), although the enzyme is not characteristically located in parenchymal cells of the adult human thyroid gland. Since homologous C cells probably give rise to medullary carcinomas in man, it is quite possible that the finding of alkaline phosphatase in cultures of tumor represents the derepression of an embryologic condition.

The reversible loss of enzyme activity after infection with an oncogenic DNA virus appears to be novel. While SV40 transformation of mouse cells is associated with a depression of enzymes responsible for ganglioside synthesis (13), such a change is not known to be reversible. Most of the data relative to virus influence on alkaline phosphatase synthesis has been obtained with RNA viruses. Infection of HeLa cells with hemadsorption type 2 virus apparently affects the gene controlling alkaline phosphatase synthesis with the subsequent loss of a specific isoenzyme (14). When one of the oncogenic RNA viruses, radiation leukemia virus of mice, causes transformation of cells, there is an actual increase of alkaline phosphatase. It is not clear whether this represents derepression of a cellular gene or expression of a viral gene (15). In the system presently described, the fact that SV40 did not increase alkaline phosphatase activity in permissive cells, but actually decreased it, was consistent with the hypothesis that SV40 may repress a cellular gene.

The increase in alkaline phosphatase in transformed cultures grown in medium supplemented with hydrocortisone is due to an as yet unknown set of factors. There is the possibility that steroid treatment aids in the selection of a portion of the total cell population which is most capable of enzyme production. In order to assess this, several efforts to clone the transformed tumor cells were made, but the plating efficiency was less than 0.1% and thus cloning experiments were not practicable in this system.

Familial medullary carcinomas of the thyroid gland are usually seen in association with pheochromocytomas of the adrenal gland and adenomas of the parathyroid gland (3). A niece of one of the patients (N. W.) whose thyroid tumor cells were studied, had a pheochromocytoma. A tissue culture line was derived from this adrenal tumor, but the uninfected cells had essentially no alkaline phosphatase activity. A number of other human tumor cell culture lines, derived from both endocrine and nonendocrine glands, were also surveyed, but again all of these in the uninfected state, exhibited no evidence of alkaline phosphatase.

Since SV40 transformed human tumor cells usually have a finite period of survival *in vitro,* present efforts are directed toward the identification of a more stable mammalian cell line which contains alkaline phosphatase and which can be permanently transformed by SV40.

Summary. Two tumors, medullary carcinomas from human thyroid glands, were used to establish tissue culture lines. These lines were assayed for alkaline phosphatase activity and were found to be consistently positive. Following transformation of the cell lines with SV40, the alkaline phosphatase levels were markedly depressed or undetectable. When hydrocortisone was added to the *in vitro* system, the enzyme activity returned to preinfection levels. This increase in alkaline phosphatase was dependent on the continuous presence of steroid; when steroid was withdrawn there was a loss of activity to pretreatment levels. No increase in enzyme levels was detected in uninfected tumor cells treated with hydrocortisone.

The authors acknowledge the skillful technical assistance of Devera E. Glazer.

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Received Sept. 17, 1971. P.S.E.B.M., 1972, Vol. 139.