Proc. Natl. Acad. Sci. USA
Vol. 77, No. 6, pp. 3464–3468, June 1980
Cell Biology

Hormonal control of human colon carcinoma cell growth in serum-free medium

(*nude* mice/primary culture/differentiation/collagen gel)

HIROKI MURAKAMI* AND HIDEO MASUI

Department of Biology, Q-058, University of California at San Diego, La Jolla, California 92093

Communicated by Nathan O. Kaplan, March 6, 1980

ABSTRACT A human colon carcinoma cell line, HC84S, was established in serum-supplemented medium from a colon tumor line T84 transplanted in *nude* mice. These cells also grew in a serum-free, synthetic medium supplemented with insulin, glucagon, epidermal growth factor, transferrin, hydrocortisone, triiodothyronine, selenium, and ascorbic acid. HC84S cells grew 3 times faster in this medium than in serum-containing medium and formed gland-like structures closely resembling the original tumor morphologically. In serum-containing medium, the cells grew as a monolayer and did not form such structures. Primary cultures from transplantable human colon tumor lines maintained in *nude* mice and a primary tumor from a patient were established directly in this hormone-supplemented medium in collagen-treated plastic dishes without fibroblast overgrowth. The hormone-supplemented medium may be generally useful for the establishment of human colon carcinoma cell lines.

With many types of cancer, the growth of cells is not totally unrestrained and may be influenced by the hormonal milieu of the host. Growth of some cancers of endocrine-responsive tissues—for example, mammary, prostate, endometrium, and thyroid—is dependent on the same hormones that control the growth and maintenance of function of the normal tissue of origin. The growth of nonendocrine normal tissue is rigidly controlled in animals, and growth of tumors in these nonendocrine tissues probably is also affected by hormones. However, there is little information about which hormones control the growth of most nonendocrine tissues and far less knowledge of the control of growth of tumors of these tissues. Recently, Sato and his colleagues (1–7) have demonstrated hormone requirements for growth of more than 30 established cell lines by growing them in hormone-supplemented serum-free media. This approach provides a method for the study of the hormonal requirements for growth of tumor cells of nonendocrine origin on the assumption that many of the hormones that stimulate or inhibit the growth of cells of a particular tumor in culture will have the same effect on that tumor in the animal.

To study the hormonal requirements for growth of a cancer, we have chosen human colon carcinoma, one of the major human cancers. These tumors can be transplanted and maintained in *nude* mice with a high rate of success. This paper reports the establishment of a human colon carcinoma cell line (HC84S) and development of a hormone-supplemented, serum-free medium (medium HC) for this cell line that permits rapid cell growth and expression of morphological characteristics similar to those found in the original tumor line (T84) in *nude* mice. Other human colon carcinoma cell lines can be established in this defined medium in collagen-coated plastic culture dishes directly from colon tumor lines transplanted in *nude* mice or from primary tumors in patients. Under these conditions, carcinoma cells grow without fibroblast overgrowth.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Transplantable Human Colon Carcinomas in *Nude* Mice. A transplantable human colon carcinoma line (T84) was established in BALB/c *nude* mice. Tumor specimens were obtained from lung metastases in a patient with colon carcinoma and injected subcutaneously into *nude* mice. The tumors produced were successfully transplanted. The original histological characteristics of these colon carcinomas were maintained throughout transplantation in *nude* mice. Their characteristics were described in detail by Reid *et al.* (8). The other transplantable human colon carcinoma lines referred to in this report were established from primary colon tumors by the same method.

Cell Culture. T84 tumor tissue from *nude* mice was minced into small pieces in a 1:1 mixture of Dulbecco–Vogt modified Eagle's medium (DME medium) and Ham's F-12 medium supplemented with 15 mM Hepes buffer, 1.2 g of $NaHCO_3$, 40 mg of penicillin, 8 mg of ampicillin, and 90 mg of streptomycin per liter, 2.5% fetal calf serum, and 5% horse serum (the mixed medium without serum is referred to as SFFD). Minced tissues from 1 g of tumor were diluted with 20 ml of culture medium and transferred to test tubes. When the large pieces of tissue, mainly connective tissue, had settled to the bottom of the tubes, the supernatant containing small aggregates of tumor cells was transferred to new tubes and the cells were collected by low-speed centrifugation. The cells were then plated in 15 culture flasks each containing 5 ml of the same medium and incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C. In a few weeks colonies of epithelial cells grew up. To kill fibroblasts, the cultures were then treated with antiserum prepared in rabbits against mouse melanoma cells. After 2–3 months of further culture, tumor cells grew to confluency. Thereafter, these tumor cells (HC84S) were cultivated in SFFD supplemented with 2.5% fetal calf serum and 5% horse serum and subcultured every 14 days by trypsinization [0.1% trypsin and 0.9 mM EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (P_i/NaCl)].

Cell Growth and Experimental Procedures. For growth assays, HC84S cells were washed with P_i/NaCl and trypsinized. When cells detached from the culture dishes, they were treated with an equal volume of 0.1% soybean trypsin inhibitor in PBS, centrifuged, washed twice with P_i/NaCl, and resuspended in serum-free SFFD. HC84S cells grew in islets and adhered tightly to culture dishes and to each other when grown more than a week. When the cells were separated into single cells by trypsinization or collagenase treatment, microscopic exami-

Abbreviations: DME medium, Dulbecco–Vogt modification of Eagle's medium; SFFD, serum-free 1:1 mixture of DME and Ham's F-12 media; HC medium, SFFD supplemented with insulin, glucagon, transferrin, epidermal growth factor, hydrocortisone, triiodothyronine, sodium selenite, and ascorbic acid; P_i/NaCl, phosphate-buffered saline; T3, triiodothyronine; EGF, epidermal growth factor; VIP, vasoinhibitory peptide.

* On leave of absence from Institute of Food Technology, Faculty of Agriculture, Kyushu University, Fukuoka 814, Japan.

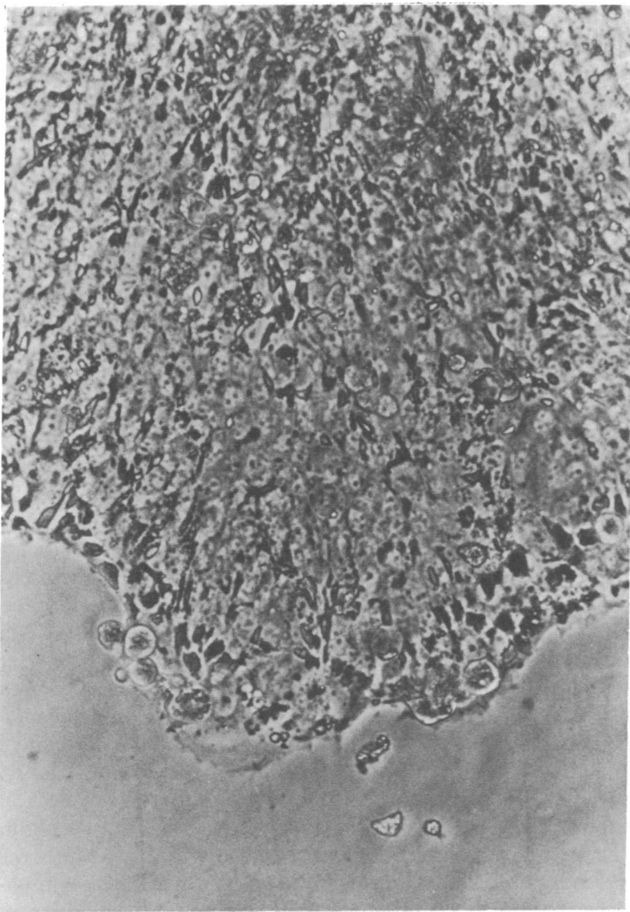

FIG. 1. Phase-contrast micrograph of HC84S cells in serum-supplemented medium. (×73.)

nation always revealed a substantial breakdown of cells. Comparisons of cell number and protein content were made for HC84S cells grown in serum-supplemented and in serum-free SFFD supplemented with hormones (HC medium). Cell number and protein content varied linearly; the mean (±SD) was 1.5 ± 0.3 $\mu g/10^3$ cells for cells grown in serum-supplemented medium and 1.2 ± 0.3 $\mu g/10^3$ cells for those grown in HC medium. For this reason we estimated cell numbers by protein content measured by Lowry's method (9) with bovine serum albumin (Sigma) as a standard.

Cells (45 μg of protein) were plated in 60-mm culture dishes (Falcon) containing 4 ml of SFFD. After overnight incubation to allow cell attachment, the medium was changed SFFD containing test supplements, and the cells were grown for 21 days. Cell growth in response to the added factors was measured by assaying protein content in the culture dishes. The dishes were washed twice with P_i/NaCl, cells were lysed in 4 ml of 0.3% Na_2CO_3/1 M NaOH, and protein was determined by Lowry's method. Values reported are means of two separate dishes.

Preparation of Anti-Mouse Cell Antiserum. Mouse melanoma cells (B16) were grown in culture and 10^7 cells were injected subcutaneously into a young adult rabbit. Three more injections were made at 1-week intervals. One week after the last injection, the rabbit was bled and serum was isolated. To test its titer against mouse cells, 3T3 and B16 cells were grown in culture dishes containing 5.0 ml of medium; then, 0.1 ml of antiserum and 0.05 ml of unimmunized rabbit serum were added and incubated overnight. All mouse cells were lysed, but human cells (WI-38 and HeLa) were not affected when tested under the same condition. Primary cultures were treated with antiserum in the same way to kill mouse fibroblasts.

Materials. Insulin, human transferrin, glucagon, hydrocortisone, triiodothyronine (T3), gastrin, and ascorbic acid were obtained from Sigma. Epidermal growth factor (EGF) was obtained from Collaborative Research (Waltham, MA). Sodium selenite was obtained from Difco. Purified bovine insulin was a gift from Eli Lilly.

RESULTS

Growth of HC84S Cells. When grown in serum-supplemented medium, HC84S cells have the typical morphological characteristics of epithelial cells (Fig. 1). When HC84S cells were injected subcutaneously into *nude* mice (10^6 cells per animal) they produced tumors in all animals, and the histological characteristics of these tumors were similar to those of the original T84 tumor line from which the HC84S line was established.

A typical growth curve of HC84S cells in serum-supplemented SFFD is shown in Fig. 2. During exponential growth the doubling time was 6–7 days. When plated at high density (40 μg of protein per dish), in SFFD in the absence of serum, the cells grew after a lag of about 9 days (Fig. 2). After this lag period, doubling time in SFFD was comparable to that in serum-supplemented medium. When cells were plated in SFFD at lower cell densities (4 μg of protein per dish or less) they did not grow.

Growth of HC84S Cells in Hormone-Supplemented Serum-Free Medium. Various hormones and growth factors were tested for their effects on growth of HC84S cells. Each was added individually to cells in SFFD (Table 1). Insulin gave the greatest stimulation of growth and showed maximal effect at 0.5 μg/ml (Fig. 3). Glucagon, transferrin, and EGF significantly stimulated growth of HC84S cells. They were maximally stimulatory at concentrations of 50 ng/ml, 0.25 μg/ml, and 0.5 ng/ml, respectively. T3, hydrocortisone, sodium selenite, and ascorbic acid also gave a significant growth enhancement.

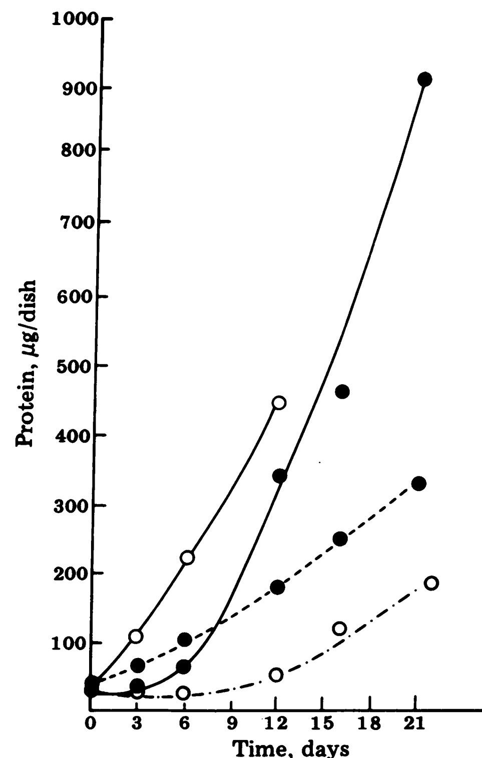

FIG. 2. Growth curves for HC84S cells in serum-supplemented medium (●·····●), serum-free medium (○-·-○), and HC medium on plastic surfaces (●—●) or on collagen gels (○—○). Points represent the averages of duplicate plates.

Table 1. Response of HC84S cells to hormones and factors

Addition	Concentration	% of growth
None (control)	—	100
Serum	7.5%	262
Insulin	2 μg/ml	234
Glucagon	200 ng/ml	157
Transferrin	2 μg/ml	152
EGF	1 ng/ml	138
T3	0.5 nM	125
Sodium selenite	25 nM	124
Ascorbic acid	10 μg/ml	119
Hydrocortisone	50 nM	118
Gastrin	50 pM	114
Vasoinhibitory peptide (VIP)	50 pM	104
Secretin	50 pM	103
Cholecystokinin (CCK)	50 pM	102
Tripeptide (Glu-His-Lys)	10 ng/ml	102
Growth hormone	1 μg/ml	102
Putrescine	0.1 mM	100
Somatostatin	10 ng/ml	93
Prostaglandin A_1	25 ng/ml	77
Prostaglandin B_1	25 ng/ml	86
Prostaglandin E_1	25 ng/ml	107
Prostaglandin E_2	25 ng/ml	93
Prostaglandin $F_2\alpha$	25 ng/ml	91
Progesterone	50 nM	83
Testosterone	50 nM	77
Estradiol	50 nM	74

The numbers represent the averages of duplicate plates.

Among intestinal hormones tested, only gastrin gave some stimulation. With the exception of prostaglandin E_1, all of the prostaglandins tested inhibited the growth of HC84S cells. Progesterone, testosterone, and estradiol also inhibited cell growth.

Because the insulin preparation used in these experiments contained glucagon as an impurity, highly purified insulin was tested to exclude the possibility that the observed insulin effect was due to glucagon. The stimulation of HC84S cell growth by highly purified insulin was comparable to that of the previously tested preparation. The glucagon preparation used contained some insulin as an impurity. However, because glucagon stimulated the growth of HC84S cells at an order of magnitude lower concentration than did insulin, it is unlikely that the effect of glucagon was due to the contaminating insulin. Thus, both insulin and glucagon stimulate the growth of HC84S cells.

HC84S cells were grown in SFFD medium supplemented with insulin (2 μg/ml), glucagon (0.2 μg/ml), transferrin (2 μg/ml), EGF (1 ng/ml), hydrocortisone (50 nM), T3 (0.5 nM), selenite (25 nM), and ascorbic acid (57 μM) (HC medium) and their growth was compared with that in serum-supplemented SFFD (Fig. 2). The cells began to grow exponentially after a lag of 3–5 days in HC medium and thereafter grew with a doubling time of about 5 days, faster than in serum-containing medium. When HC84S cells were plated on collagen gels in the hormone-supplemented, serum-free medium, they grew without a lag period. HC84S cells grown in this hormone-supplemented, serum-free medium piled up and produced gland-like structures (Figs. 4 and 5) morphologically closely resembling those of T84 tumors in animals. This appearance is strikingly different from the monolayers formed by HC84S cells in serum-supplemented medium (Fig. 1).

Establishment of Other Human Colon Cancer Cell Lines in Hormone-Supplemented Medium. We wished to determine whether cells taken from T84 tumors maintained in *nude* mice could be established directly in the defined medium developed for HC84S cells. Primary cultures were prepared from a T84 tumor and placed in serum-supplemented SFFD overnight. The next day the medium was replaced with the serum-free HC medium. The cells grew in this medium at a rate comparable to that in serum-containing medium. The substances found to be the major growth promotors for HC84S cells were examined individually for their effects on growth of primary T84 cells (Fig. 6). Among the factors tested singly, transferrin showed the most striking growth-promoting effect.

Fibroblast overgrowth is a problem often encountered in the

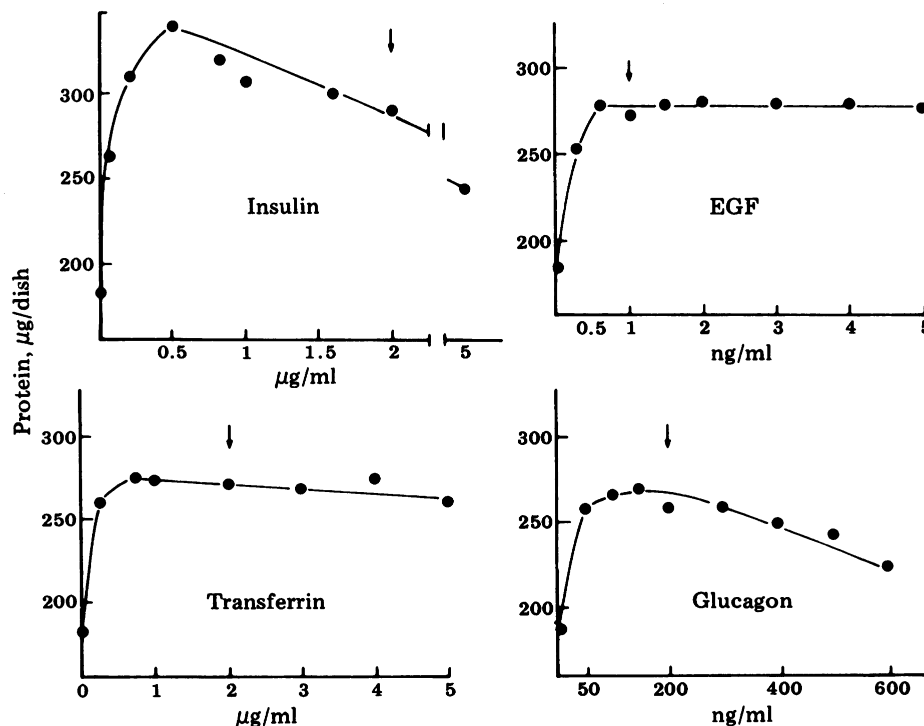

FIG. 3. Dose–response curves of HC84S cell growth to insulin, transferrin, EGF, and glucagon. Points represent the averages of duplicate plates.

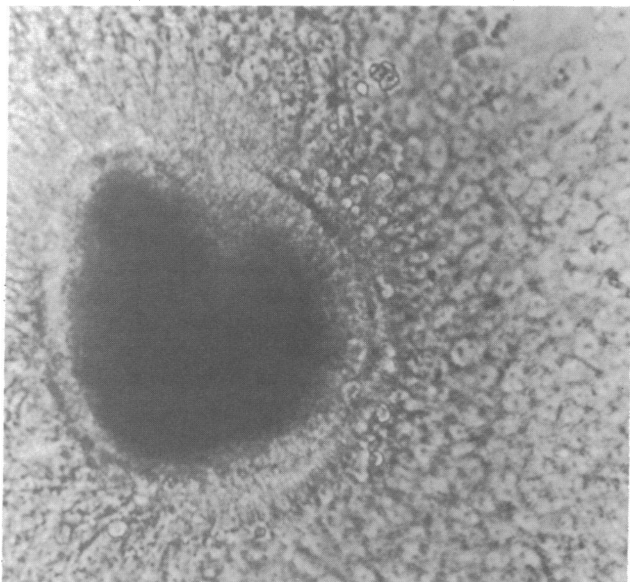

FIG. 4. Phase-contrast micrograph of HC84S cells in HC medium. (×73.)

establishment of primary cell cultures. Primary T84 cells were tested for growth in Falcon plastic culture dishes with or without a collagen gel coating in serum-free or serum-containing media to find optimal culture conditions. T84 cells attached more quickly to collagen-coated dishes than to uncoated ones irrespective of the growth medium. Overgrowth of T84 cells by fibroblasts was a problem in cultures in the uncoated plastic flasks where the T84 cells took longer to become established. Fibroblasts grew better in the cultures with serum supplementation than in HC medium. Thus, of the four growth conditions, the optimal one for establishment of primary cultures is hormone-supplemented serum-free medium and collagen-coated dishes.

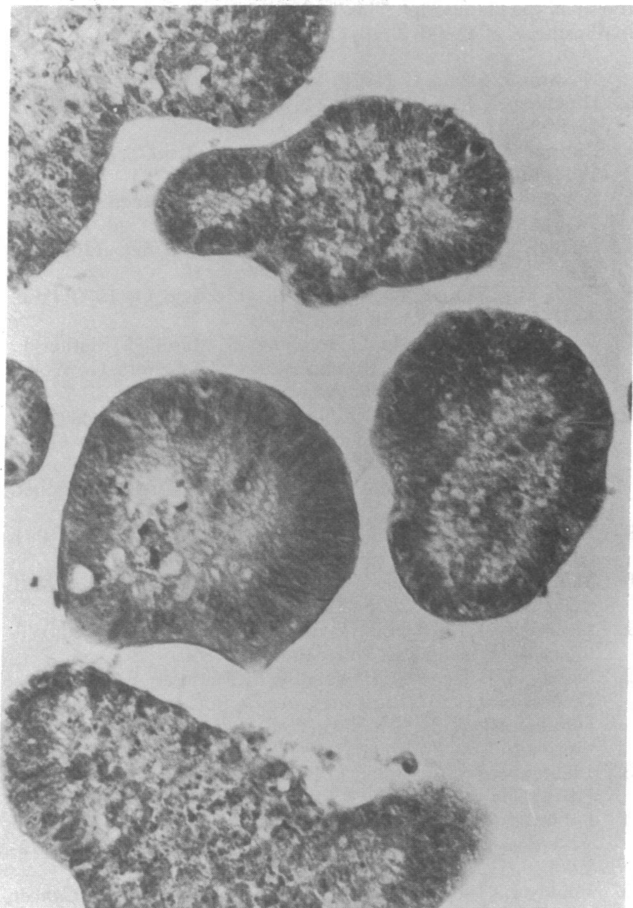

FIG. 5. Thin section of gland-like structures formed by HC84S cells in HC medium. Cells were harvested, fixed in 10% formalin, and embedded in 0.5% agar. The agar was dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. (×73.)

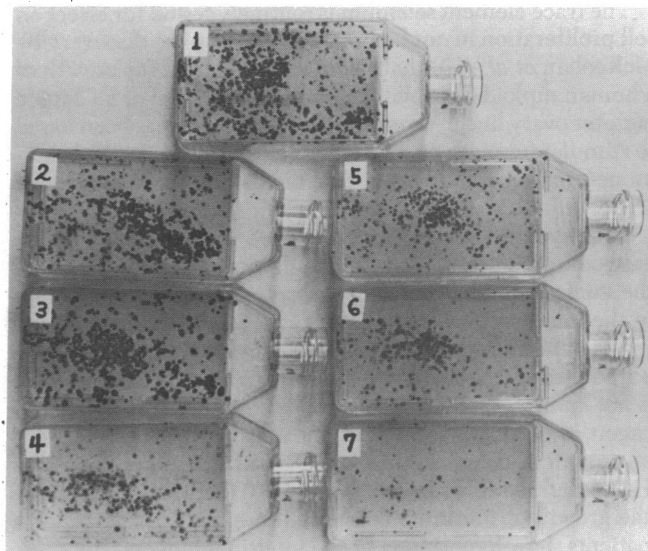

FIG. 6. Response of primary cultures from a T84 tumor to hormones and factors. After 21 days, the cultures were fixed with 10% formalin and stained with crystal violet. Bottles: 1, serum-supplemented medium; 2, HC medium; 3, SFFD + transferrin; 4, SFFD + insulin; 5, SFFD + EGF; 6, SFFD + glucagon; 7, SFFD.

These observations suggested that the use of collagen-coated dishes and the hormone-supplemented serum-free medium developed for HC84S cells might allow establishment of other cell lines from human colon carcinomas. As a test, primary cultures were prepared from two human colon carcinomas (T219 and T245) transplanted in *nude* mice and from a carcinoma taken directly from a patient and successfully cultured under these conditions. The cells from T219 and T245 have been passaged eight times and the cells directly from a patient have been passaged three times.

DISCUSSION

A human colon carcinoma cell line (HC84S) has been established in culture from a tumor line (T84) transplantable in *nude* mice. Insulin, glucagon, transferrin, EGF, hydrocortisone, T3, selenite, ascorbic acid, and gastrin were found to stimulate growth of these cells in serum-free medium. When these supplements, with the exception of gastrin, were added to SFFD, to form HC medium, the HD84S cells grew 3 times faster than in serum-containing medium and exhibited a gland-like appearance similar to that of T84 tumors maintained in mice. [Gastrin was not tested for stimulation of HC84S cell growth (it was only mildly stimulatory) until after the experiments with HC medium had been completed.] Sato and coworkers have determined hormone requirements for a large number of cells lines (1–7). Each cell line requires a different set of hormones, with some components such as insulin and transferrin being common to most but others being rather specific. Glucagon and gastrin, which promote growth of HC84S cells, have not been identified as requirements for growth of the other lines. An important unanswered question is whether the hormones that stimulate the growth of human colon carcinoma cells in culture stimulate them in animals. This can now be tested with *nude* mice carrying human colon carcinomas.

The trace element selenium is routinely tested for effect on cell proliferation in our laboratory following the discovery by McKeehan *et al.* (10) that selenium is essential for growth of a human diploid fibroblast cell line (WI-38) and of a Chinese hamster ovary line in serum-depleted media. It has been found to stimulate growth of a number of cell lines for which hormone-supplemented, serum-free media have been developed (7).

Glucagon has been reported to have various effects on the gastrointestinal tract including inhibition of acid secretion in the stomach and inhibition of absorption of water and sodium in the intestine (11). Enteroglucagon, a substance that cross-reacts immunologically with pancreatic glucagon, is known to be secreted by the small intestine although its physiological role is not known (12). The results reported here suggest that glucagon, or possibly enteroglucagon, may be involved in control of growth of colon cell *in vivo*. Gastrin stimulates growth of HC84 slightly. It has been reported (13) that gastrin stimulates the growth of intestinal cells in culture and that gastrinoma patients show hyperplasia of the intestinal mucosa. Recently, Laburthe *et al.* (14) reported that vasoinhibitory peptide (VIP) activates adenyl cyclase of a human colon carcinoma cell line (HT 29) but the effects of VIP on growth of these cells were not studied. We found VIP not to affect growth of HC84S cells. These results raise the possibility that some hormones that do not affect cell growth nevertheless do affect cell function. It remains to be elucidated whether any of the hormones found to stimulate growth of HC84S cells affect their function, but this is one possible interpretation of the result that HC84S cells apparently differentiate morphologically when grown in hormone-supplemented, serum-free medium.

Somatostatin, prostaglandins (except prostaglandin E_1), progesterone, testosterone, and estradiol appeared to inhibit growth of HC84S cells. This result and the demonstration that a hormone-supplemented, serum-free medium allows better growth of HC84S cells than does serum-containing medium raise the possibility that there are hormones present in serum that inhibit the growth of human colon carcinoma cells. It may be possible to develop combinations of hormones that effectively inhibit HC84S cell growth in just the way that the growth medium was constructed.

Some of the hormones present in serum may inhibit HC84S cell differentiation of function because the cells grew in three dimension and formed gland-like structures similar to those of T84 tumors in animals when put in hormone-supplemented medium whereas they grew as a monolayer in serum-containing medium. Recently, Taub *et al.* (6) reported that hemicyst formation by MDCK dog kidney cells was similarly dependent on the hormone components of the medium.

Human colon carcinoma cell lines could be established more easily on collagen gels than on a plastic surface. Our observation that HC84S cells started to grow in defined medium on collagen gels without a lag period after plating suggests that the collagen may facilitate formation of a cell–substrate adhesion that is an essential prerequisite for cell division. An advantage of the collagen supports is that cell lines could be established from a tumor before fibroblast overgrowth became a problem. This problem has made it difficult to grow epithelial cells in culture in the past.

The hormone-supplemented medium described here supported the growth of all the tumor cells tested, including those taken directly from a patient. The fact that primary cells from a T84 tumor gave the greatest growth response to transferrin rather than to insulin, which was the single most effective stimulatory of HC84S cells derived from another T84 tumor, suggests that cells from different colon tumors will have somewhat different hormonal requirements. Hence, the medium developed for HC84S cells may not be optimal for growth of all human colon carcinoma lines, but it appears to be generally applicable.

By using medium in which hormones and factors have replaced serum, the mechanisms by which human colon carcinomas grow and differentiate become accessible to dissection. Carcinogenesis is a multistep process. Once cancers are established in animals, they undergo a series of changes in their properties known as tumor progression (14–16). We maintain several human colon carcinomas by transfer in *nude* mice. These originated in different patients with different extents of metastasis, and they differ in their histological characteristics and growth rates in *nude* mice. Thus, they represent different degrees of malignancy. Because cell lines from these can be established in defined medium and their hormone requirements determined, it may be possible to correlate hormone requirements with various states of malignancy and to study the processes involved in establishing malignancy. HC medium was developed to support growth of a cell line derived from a lung metastasis of a colon carcinoma, and it also supported growth of three other colon carcinomas. However, the colon primary tumor that gave rise to the lung metastasis was not available to us for a detailed comparison of hormonal requirements and correlation with tumor progression.

We thank Terry White and Bonnie Wolfe for excellent technical assistance, Drs. Nathan O. Kaplan and Gordon Sato for advice and encouragement, and Patricia Romans and Dr. David Barnes for their criticism of the manuscript. This investigation was supported by National Institutes of Health Grant CA-23052.

1. Hayashi, I. & Sato, G. (1976) *Nature (London)* **259,** 132–134.
2. Hutchings, S. E. & Sato, G. H. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 901–904.
3. Rizzino, A. & Sato, G. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1844–1848.
4. Bottenstein, J. & Sato, G. H. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 514–517.
5. Mather, J. P. & Sato, G. H. (1979) *Exp. Cell Res.* **120,** 191–200.
6. Taub, M., Chuman, L., Saier, M. H., Jr. & Sato, G. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3339–3342.
7. Bottenstein, J., Hayashi, I., Hutchings, S., Masui, H., Mather, J., McClure, D., Ohasa, S., Rizzino, A., Sato, G., Serrero, G., Wolfe, R. & Wu, R. (1979) *Methods Enzymol.* **59,** 94–109.
8. Reid, L., Holland, J., Jones, C., Wolfe, B., Niwayama, G., Williams, R., Kaplan, N. & Sato, G. (1978) in *Proceedings of the Symposium on the Use of Athymic (Nude) Mice in Cancer Research*, eds. Houchens, D. & Overjera, A. (Gustav, Fischer, NY), pp. 107–121.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, P. J. (1951) *J. Biol. Chem.* **193,** 265–275.
10. McKeehan, W. L., Jones, W. G. & Ham, R. G. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2023–2027.
11. McGuigan, J. E. (1974) in *Textbook of Endocrinology*, ed. Williams, R. H. (Saunders, Philadelphia), p. 850.
12. Moody, A. J., Jacobsen, H., Sundby, F., Frandsen, E. K., Baetens, D. & Orci, L. (1977) in *Glucagon: Its Role in Physiology and Clinical Medicine*, eds. Foá, P. P., Bajaj, J. S. & Foá, N. L. (Springer, New York), pp. 129–136.
13. Lichtenberger, L., Miller, L. R., Erwin, D. N. & Johnson, L. R. (1973) *Gastroenterology* **65,** 242–251.
14. Laburthe, M., Rousset, M., Boissard, C., Chevalier, G., Zwebaum, A. & Rosselin, G. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2772–2775.
15. Foulds, L. (1964) in *Cellular Control Mechanisms and Cancer*, eds. Emmelot, P. & Mühlbock, O. (Elsevier, Amsterdam), pp. 242–258.
16. Medina, D. (1975) in *Cancer*, ed. Becker, F. F. (Plenum, New York), Vol. 3, pp. 99–119.