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Growth of Human Small Cell (Oat Cell) Carcinoma of the Lung in Serum-free Growth Factor-Supplemented Medium

Elisabeth Simms, Adi F. Gazdar, Paul G. Abrams, and John D. Minna[1]

National Cancer Institute-Veterans Administration Medical Oncology Branch, Division of Cancer Treatment, National Cancer Institute, and Veterans Administration Medical Center, Washington, D. C. 20422

ABSTRACT

A cell culture line of human small cell (oat cell) carcinoma of the lung (NCI-H69) was studied for growth factor requirements for replication in serum-free medium. By a series of addition experiments, the combination of selenium (3×10^{-8} M), hydrocortisone (10^{-8} M), insulin (5 μg/ml), transferrin (100 μg/ml), and 17β-estradiol (10^{-8} M) (SHITE) added to Roswell Park Memorial Institute Medium 1640 without serum was found to allow optimum replication when cells were transferred from serum-containing medium. NCI-H69 cells have replicated continuously in SHITE-supplemented Roswell Park Memorial Institute Medium 1640 for periods of more than 12 months. The cells replicate with approximately the same doubling times as in medium supplemented with 10% fetal calf serum, but they exhibited a lower saturation density and longer lag phase in SHITE-supplemented medium compared to serum-supplemented medium. When individual components of SHITE were deleted, immediate and dramatic differences in growth were seen only with deletion of transferrin while minimal decreases were noted with deletion of the other factors. After 6 months of growth in SHITE, deletion of insulin or transferrin from the formula resulted in cessation of cell growth in serum-free medium while deletion of hydrocortisone or estradiol (alone or together) did not. A variety of other hormones and growth factors did not add significantly to SHITE in promoting replication. In addition, a series of hormones was found to inhibit replication or saturation density achieved in serum-free, hormone-supplemented medium including epidermal growth factor, luteinizing hormone-releasing factor, parathyroid hormone, blood meal, and the tripeptide glycyl-L-histidyl-L-lysine acetate. Six other small cell carcinoma lines were tested and also were able to replicate in SHITE-supplemented serum-free medium indicating the generality of this formula for small cell carcinoma. NCI-H69 cells cultured for 4 months in SHITE medium retained their amine precursor uptake and decarboxylation properties, characteristic small cell carcinoma histology by light microscopy, dense core (neurosecretory) granules by electron microscopy, and tumorigenicity in nude mice. The identification of this growth formula should allow selection of tumor cells directly from patient samples, study of hormone production and regulation by these cell lines, and identification of new growth and therapeutic agents, and it should provide a way of selecting for normal amine handling cells related histogenetically to small cell carcinoma.

INTRODUCTION

Small cell carcinoma of the lung represents 20 to 25% of lung cancer occurring in the United States and has distinct clinical, biological, and morphological properties (10, 12). This tumor type, in contrast to other lung cancers, responds dramatically to chemotherapy and radiotherapy and in fact may be cured by these modalities when in a surgically unresectable state (23). In addition, it is often associated with paraneoplastic syndromes and the production of a variety of hormones including ACTH,[2] AVP, and calcitonin (27, 29). Small-cell carcinomas are believed to be derived from cells of the diffuse neuroendocrine system portion of the APUD (24, 25, 31). In trying to develop new methods of treating this tumor, we have derived continuously replicating, clonable cell lines and characterized them as small cell carcinomas (17). The identification of hormones and growth factors required for the replication of such cells would be of potential therapeutic use and of interest in the study of the biology of the APUD system. We have followed the lead of Hayashi and Sato (20) and studied the replication of our small cell carcinoma of the lung cell lines in serum-free media supplemented with a variety of growth factors and hormones. In this paper, we report that the combination SHITE will permit the continuous replication for over 12 months of small cell carcinoma of the lung *in vitro*, as well as the continued expression of differentiated APUD functions.

MATERIALS AND METHODS

Cell Lines. The human small cell carcinoma of the lung tissue culture lines were adapted to culture by us either directly from our patients (H designation, *e.g.*, NCI-H69) or from transplantable nude-mouse tumors (N designation, *e.g.*, NCI-N230). The details of their establishment and characterization are reported elsewhere (17). All of the lines came from patients with a histological and clinical diagnosis of small cell carcinoma of the lung, were tumorigenic in nude mice, had human enzymes on starch gel electrophoresis (19), had high specific activities of L-aromatic amino acid decarboxylase [L-dopa decarboxylase (EC 4.1.1.2.8)], demonstrated formaldehyde-induced fluorescence, showed neurosecretory granules by electron microscopy, and had histology and cytology typical of small cell carcinoma. They were continuously growing cell lines that

[1] To whom requests for reprints should be addressed, at National Cancer Institute-Veterans Administration Medical Oncology Branch, Veterans Administration Medical Center, 50 Irving Street, N. W., Washington, D. C. 20422.

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[2] The abbreviations used are: ACTH, adrenocorticotrophin; AVP, arginine vasopressin; APUD, amine precursor uptake and decarboxylation series; SHITE, selenium:hydrocortisone:insulin:transferrin:17β-estradiol; RPMI, Roswell Park Memorial Institute; FCS, fetal calf serum; T3, triidothyronine; LHRF, luteinizing hormone-releasing factor; PTH, parathyroid hormone; TRH, thyrotropin-releasing hormone; EGF, epidermal growth factor; NGF, nerve growth factor; IT, insulin:transferrin; HIT, hydrocortisone:insulin:transferrin; ITE, insulin:transferrin:17β-estradiol; HITE, hydrocortisone:insulin:transferrin:17β-estradiol.

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could be cloned in microwells and in 0.3% agarose. Stock cultures of NCI-H69 were typical of these, being a floating line which grows in balls, clumps, and chains in a suspension culture. They were maintained in RPMI Medium 1640 supplemented with 10% (v/v) FCS in 75-sq cm Falcon flasks. It was derived from the malignant pleural effusion of a male patient and required conditioned medium from another small cell carcinoma line (NCI-H45) for its establishment. After 6 months in conditioned medium, it could be shifted to unconditioned medium and were maintained in this prior to the current study. The subline of NCI-H69 used in the current study was free of mycoplasma contamination by bacteriological tests done at Microbiologic Associates, Bethesda, Md. and was aneuploid by flow microfluorometric analysis with a DNA index of 1.75 (compared to a diploid human lymphocyte standard) by Dr. P. Bunn of our laboratory. RPMI Medium 1640 and modified F12 medium (called F14 medium) (13) were prepared from powder obtained from Grand Island Biological Co., and liquid RPMI Medium 1640 from Grand Island Biological Co. was also used. Water for tissue culture medium was prepared using a Millipore "Super-Q" system. Falcon and Costar plastic ware was used, and cultures were maintained in a water-jacketed incubator with 100% humidity and 95% air:5% CO_2 atmosphere.

Growth Factor and Hormones. Growth factors and hormones were obtained from the following sources. From Sigma Chemical Co., St. Louis, Mo. were: ACTH, porcine, Grade 1 (Lot 37C-0138); 17β-estradiol (Lot 178-0287); follicle-stimulating hormone, porcine (Lot 106C-0244); crystalline glucagon, bovine and porcine pancreas (Lot 77C-0320); hydrocortisone (Lot 71C-0020); insulin, bovine pancreas crystalline (Lots 121C-1350 and 66C-0395, 26.4 IU/mg); progesterone (Lot 95C-0320); prolactin (luteotrophic hormone) from sheep pituitary (Lot 127C-0182); putrescine dihydrochloride (Lot P 7505); T3 (Lot 95C-0388); testosterone acetate (Lot 73C-1210); transferrin, human over 90% iron free (Lot 96C-0107); and Pitressin. From Calbiochem-Behring Corp., La Jolla, Calif. were: calcitonin, porcine (Lot 720117); glycyl-L-histidyl-L-lysine acetate, Grade A, (Lot 600700); luteinizing hormone, equine, Grade B (Lot 602250, 1.26 Armour units/mg); LHRF, synthetic (Lot 760003); PTH, bovine (Lot 700667); somatostatin, synthetic (Lot 628094); and TRH, synthetic (Lot 600924). From Collaborative Research, Waltham, Mass. were: EGF, mouse submaxillary gland (Lot 794-5); fibroblast growth factor, bovine pituitary gland (Lot 794-67D); multiplication-stimulating activity, rat liver cell (Lot 734-27); and NGF, mouse submaxillary gland (Lot 794-70). From other sources, we had gifts of: growth hormone, human (National Pituitary Agency, distributed by NIAMDD, Lot HS2160E); thyrotropin, bovine (Miles Laboratories, Elkhart, Ind., via Dr. L. Kohn, NIH Lot 33); and blood meal, bovine, crude extract (Dr. G. Sato). Stock solutions of the hormones and growth factors were prepared at a concentration of 100 to 2500 times the final concentration. The procedure of preparing these hormone concentrates varies with each compound. The steroid hormones were dissolved in ethanol and then diluted in 0.9% NaCl solution while glucagon, T3, and insulin required adjustments of the pH to go into solution. The highly concentrated stock solutions were then filter sterilized (0.2-μM Nalgene filter) and stored at −20° in 1- to 3-ml aliquots in glass vials. ACTH and insulin were stored at 4°. For each experiment, the appropriate factors were thawed and diluted to 100 times their final concentration in serum-free medium, and then, appropriate volumes of the 100-fold concentrates were mixed together to make the various experimental combinations at a final 2-fold concentration. One ml of the 2-fold-concentrated medium was then plated into each well of a Costar 24-well plate according to the experimental design, and the plates were placed in a tissue culture incubator to equilibrate temperature and pH prior to addition of 1 ml of cell suspension.

Growth Curve Experiments. Growth curves were performed in duplicate in 24-well (16 mm diameter) cluster plates. A cell suspension was prepared by collecting cells by centrifugation from stock medium containing cells growing in suspension in active growth phase. The cells were washed twice with serum-free medium (either RPMI Medium 1640 or F14) and suspended by trituration in a 10-ml Falcon tissue culture plastic pipet. Viable cell count was determined by trypan blue exclusion in a hemocytometer, and appropriate dilutions of viable cells were made to give a final concentration of 5×10^4 viable cells/ml. One ml of this cell suspension was then added to each well into which previously had been placed 1 ml of the test medium with a 2-fold concentration of growth factors or hormones so that the final concentration was as reported in the results. Wells were fed with 0.5 ml of medium containing the final hormone concentration every 3 to 4 days, and after the first feeding, 0.5 ml of spent medium was removed from each well prior to feeding. Cell counts were performed at the indicated intervals by removing the entire contents of one well with a Pasteur pipet, washing the well once with phosphate-buffered saline (Grand Island Biological Co., Grand Island, N. Y.), centrifuging the cells, discarding the supernatant and resuspending the cells in a known volume of either RPMI Medium 1640 or F14 medium, and counting the suspension in a hemocytometer. The data represent the average of duplicates of total cell numbers per well determined by multiplying the cell concentration times the volume resuspended. For long-term growth after 24 days, cells were transferred to new wells in the appropriate test medium at a concentration of 5 to 10×10^4, or into larger flasks.

Other Methods. Glutaraldehyde-fixed cell pellets were examined by electron microscopy for dense core granules as described (17). L-Aromatic amino acid decarboxylase (L-dopa decarboxylase, EC 4.1.1.28) activity was determined by Dr. S. Baylin (Johns Hopkins Cancer Center) as described (2). Formaldehyde-induced fluorescence was determined by growing replicate cultures in SHITE medium, SHITE medium supplemented with 5-hydroxy-L-tryptophan (100 μg/ml), or 1-β-3,4-dihydroxyphenylalanine for 18 hr. as described (16). Tumorigenicity was determined by injecting 10^7 cells into each of 3 athymic nude mice (*nu/nu*), BALB/c background (ARS/Sprague-Dawley, Madison, Wis.).

RESULTS

Replication of Small Cell Carcinoma Line NCI-H69 in Various Concentrations of Serum. When NCI-H69 cells from moderately dense cultures were dispersed from clusters and plated at a density of 5×10^4 viable cells/2-sq cm surface area well, the cells remained in a lag phase of growth for 3 to 5 days and then began replicating with a population doubling time of approximately 48 hr in medium supplemented with 1 or 10% FCS (Chart 1). Only near the end of the growth curve,

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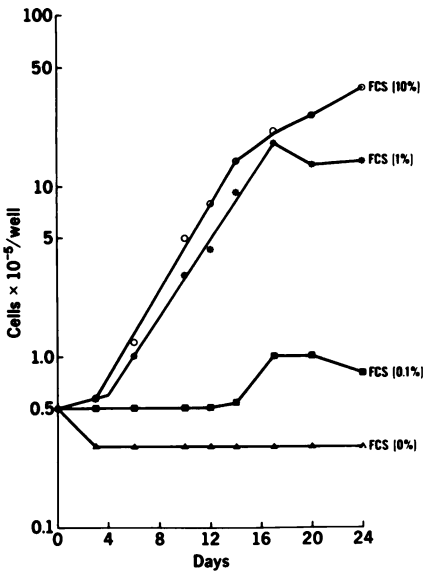

Chart 1. Growth curve of NCI-H69 cells in RPMI Medium 1640 supplemented with various concentrations of FCS.

when densities of cells of 2×10^6/well were reached, was the effect of the higher serum concentration noted. The lag phase appears to be a function of breaking up the large cell clusters (10 to 100 cells/cluster) which small-cell carcinoma tumor cells form *in vitro*. Cells plated into medium with 0.1% or no serum maintained the cell number for long periods (3 to 5 weeks) and sometimes underwent a limited number of divisions in the serum-free medium (Table 1; Charts 1 and 2). In a series of experiments, when 5×10^4 NCI-H69 cells were plated on Day 0 and then maintained in a serum-free medium, 1, 3, 7, 11, 11, 20, and 35×10^4 cells were found on Days 21 to 36 in 7 separate tests. This replication often occurred toward the end of the growth curves after an initial rise and fall and then subsequent rise in cell number. This could not be correlated with feeding of the cultures. However, after 6 weeks in serum-free medium, growth ceased. Because of the lag phase with cluster dispersal and because we were interested in developing a medium that supported the long-term continuous replication of small cell carcinoma in serum-free medium, we attempted to maintain stock cultures in RPMI Medium 1640:10% FCS in the log phase of growth as single cells and small clusters. We then assayed cell numbers at different times during the log and stationary phases of growth in parallel cultures of those cells in medium supplemented with FCS, hormones, and growth factors or no additions.

Sequential Studies of the Growth Factor Requirements of NCI-H69 Cells. We began by adding insulin (10 μg/ml) and transferrin (5 μg/ml) to serum-free medium as this IT combination appeared to be a common requirement from other studies of growing cells in serum-free medium (5) (Table 1). There was significant growth in IT-supplemented medium alone. Increasing the concentration of transferrin 20 fold to 100 μg/ml gave an additional stimulation of high cell density to IT medium (Table 1). For most future experiments, this amount of transferrin was used except with Medium F14 where transferrin (100 μg/ml) was inhibitory compared with 5 μg/ml, and the latter concentration was used. F14 contains iron while RPMI Medium 1640 does not. When hydrocortisone (10^{-8} M) or 17β-estradiol (10^{-8} M) was added to the combination IT (HIT or ITE), a slight stimulation of high cell density was seen (Table 1). When the components of HITE were mixed together, additional growth over the use of HIT alone was seen (Table 1;

Table 1

Growth of NCI-H69 cells in serum-free and hormone-supplemented RPMI Medium 1640 in low density (log phase) and high cell density growth conditions

Additive	Relative no. of cells[a] Low density	High density
Experiment 1	*Day 12*	*Day 20*
None (RPMI Medium 1640 alone)	1 (0.5×10^4)[b]	1 (2×10^4)
FCS (10%)	155	131
FCS (1%)	87	67
FCS (0.1%)	9.8	5
Insulin (10 μg/ml) + transferrin (5 μg/ml)	57	32
Insulin (10 μg/ml) + transferrin (5 μg/ml) + hydrocortisone (10^{-8} M)	52	42
Experiment 2	*Day 10*	*Day 21*
None	1 (8×10^4)	1 (7×10^4)
FCS (10%)	14	52
Insulin (10 μg/ml) + transferrin (5 μg/ml)	7	16
Insulin (10 μg/ml) + transferrin (100 μg/ml)	6.8	20
Insulin (10 μg/ml) + transferrin (100 μg/ml) + 17β-estradiol (10^{-8} M)	5.6	23
TRH (10^{-9} M)	5.5	21
T3 (3×10^{-10} M)	5.4	19
Progesterone (10^{-8} M)	5.9	17
Experiment 3	*Day 18*	*Day 24*
None	1 (18×10^4)	1 (11×10^4)
FCS (10%)	7.3	42
Insulin (10 μg/ml) + transferrin (100 μg/ml) + hydrocortisone (10^{-8} M)	3.6	15
Insulin (10 μg/ml) + transferrin (100 μg/ml) + hydrocortisone (10^{-8} M) + 17β-estradiol (10^{-8} M)	4.1	22

[a] The relative number of cells represents the ratio number of cells in experimental wells:number of cells in unsupplemented RPMI Medium 1640 wells.

[b] Numbers in parentheses, number of cells in control wells with unsupplemented medium. Cell numbers are averages of duplicate values. On Day 0, 5×10^{-4} cells were plated.

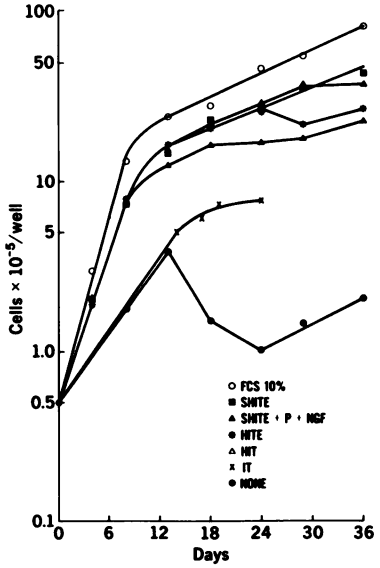

Chart 2. Growth curve of NCI-H69 cells in RPMI Medium 1640 supplemented with various additives. *P*, putrescine. Factor concentrations were listed in Table 2.

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Chart 2). The complete growth curves for some representative experiments are given in Chart 2. However, the addition of TRH (10^{-9} M), T3 (3×10^{-10} M), or progesterone (10^{-8} M) gave no significant increase over IT alone.

Effects of Addition of Other Hormones and Growth Factors to Serum-free Medium Supplemented with HITE on the Growth of NCI-H69 Cells. We also tested the supplements in F14 medium (Table 2), but dramatic growth stimulation was not seen with HIT or HITE alone. However, when selenium (2×10^{-8} M) was added to F14:HITE medium (SHITE), dramatic growth was noted. Inhibition of growth in F14:SHITE medium was seen when putrescine (10^{-4} M) and NGF (10 ng/ml) were added. This inhibition was not found in RPMI Medium 1640: SHITE medium (Chart 2). F14 already contains putrescine at a concentration of 10^{-6} M, but this would not seem to add significantly to the 10^{-4} M level of putrescine added.

A series of other growth factors and hormones was added to RPMI Medium 1640 and F14 medium supplemented with HITE (Table 3). In repeated tests, the one consistent addition to HITE which gave growth stimulation was selenium (3×10^{-8} M), previously noted to be a requirement for long-term growth of cell lines in serum-free medium (22). The effect of selenium was usually noted after 24 or more days of growth in serum-free hormone-supplemented RPMI Medium 1640 (Chart 3), but it was noticeable earlier in F14-based medium. None of the other factors gave a dramatic, consistent stimulation of cell number. However, calcitonin (0.1 ng/ml), luteinizing hormone (500 ng/ml), Pitressin (10 ng/ml), NGF (10 ng/ml), and growth hormone (5 mg/ml) gave slight stimulation in one or the other base media either during low- or high-density growth phases, and most of these were studied further.

In addition, a number of compounds appeared to inhibit the growth of cells in serum-free HITE-supplemented medium (Table 3; Chart 3). At higher cell density, an inhibitory effect in RPMI Medium 1640:HITE was seen with blood meal (10 μg/ml), EGF (1 ng/ml), Gly–His–Lys (200 ng/ml), LHRF (10 ng/ml), and PTH (0.5 ng/ml).

Table 2

Growth of NCI-H69 cells in serum-free F14 (F12 modified) medium supplemented with growth factors at low (log phase) and high cell density

Additive[a]	Relative cell no.[b]	
	Low density (Day 8)	High density (Day 24)
None (F14 alone)	1 (5×10^4)[c]	1 (11×10^4)
FCS (10%)	11	22
H + I + T	2.0	1.5
H + I + T + E	2.8	1.7
H + I + T + E + S	5.4	16
H + I + T + E + S + P + NGF	2.6	6.7

[a] H, hydrocortisone (10^{-8} M); I, insulin (10 μg/ml); T, transferrin (5 μg/ml, optimal for F14); E, 17β-estradiol (10^{-8} M); S, selenium (3×10^{-8} M); P, putrescine (10^{-4} M); NGF (10 μg/ml).

[b] Ratio of number of cells in supplemented medium:number of cells in unsupplemented F14 medium. On Day 0, 5×10^4 cells plated.

[c] Numbers in parentheses, numbers of cells in unsupplemented medium; average of triplicate values.

Table 3

Effect of addition of various hormones and growth factors to RPMI Medium 1640 or F14 medium supplemented with HITE on the growth of NCI-H69 cells

	Relative cell no.[a]		
	RPMI Medium 1640		F14
	Low density (Day 5)	High density (Day 21)	Low density (Day 15)
H + I + T + E[b]	1 (5.2×10^5)[c]	1 (15×10^5)	1 (2.5×10^5)
FCS (10%)	1.48	2.4	7.4
Additive to HITE			
Selenium (3×10^{-8} M)	1.0	1.4	4.0
ACTH (5 μU/ml)	0.79	0.97	1.0
Blood meal (10 μg/ml)	0.69	0.64	
Calcitonin (0.1 ng/ml)	1.23	0.84	1.1
EGF (1 ng/ml)	0.92	0.58	
EGF (50 ng/ml)	0.79	0.47	
FGF[d] (50 ng/ml)	0.94	0.77	
GH (5 ng/ml)	1.3	0.81	0.84
Glucagon (500 ng/ml)	0.73	0.82	
Gly–His–Lys (200 ng/ml)	0.85	0.33	
LH (500 ng/ml)	1.1	1.2	
LHRF (10 ng/ml)	0.77	0.45	
MSA (50 ng/ml)	1.0	0.70	
NGF (10 ng/ml)	1.0	0.83	1.32
NGF (50 ng/ml)	0.77	0.89	
Pitressin (10 ng/ml)	0.77	0.76	1.40
Progesterone (10^{-8} M)			0.88
Prolactin (10 ng/ml)	0.73	0.82	
PTH (0.5 ng/ml)	1.0	0.48	
Putrescine (10^{-4} M)	1.0	1.1	0.76
Testosterone (10^{-8} M)			0.88
TSH (5 ng/ml)			0.84
T3 (3×10^{-10} M) + TRH (10^{-9} M)			0.76

[a] Ratio of the number of cells in the HITE-supplemented medium plus the indicated factor:HITE-supplemented medium alone during the low-density (log) phase of growth ($<1 \times 10^6$ cells/well) and the high-density phase of growth ($>1 \times 10^6$ cells/well).

[b] The contents and concentration of HITE components were: H, hydrocortisone (10^{-8} M); I, insulin (10 μg/ml); T, transferrin (100 μg/ml for RPMI Medium 1640 and 5 μg/ml for F14); E, 17β-estradiol (10^{-8} M).

[c] Numbers in parentheses, numbers of cells in the control wells with HITE-supplemented medium only. On Day 0, 5×10^4 cells were plated.

[d] FGF, fibroblast growth factor; GH, growth hormone; LH, luteinizing hormone; MSA, multiplication-stimulating activity; TSH, thyrotropin.

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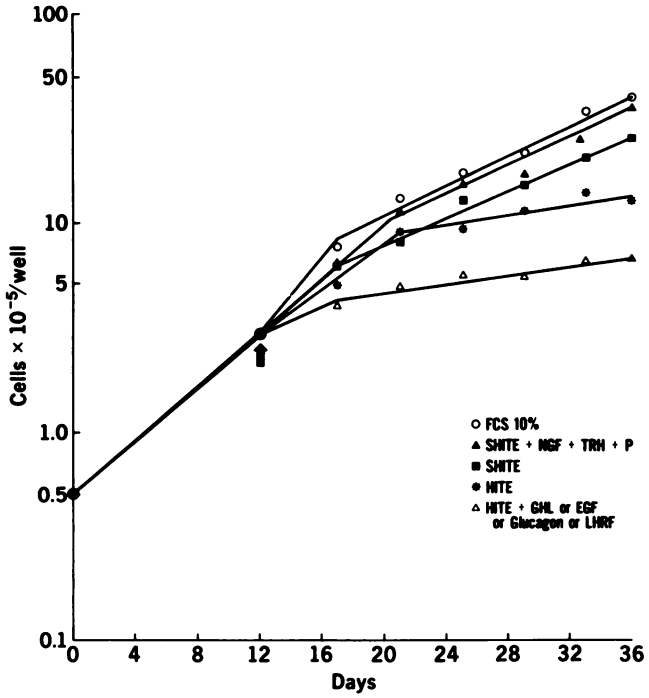

Chart 3. Growth curve of NCI-H69 cells. Cells were plated in RPMI Medium 1640 supplemented with HITE on Day 0 and switched to the indicated medium on Day 12. Hormone concentrations are listed in Table 3. NGF was tested at 10 ng/ml; EGF was tested at 1 ng/ml. *P*, putrescine; *GHL*, glycyl-L-hystidyl-L-lysine acetate.

Search for Other Factors to Augment the Growth of NCI-H69 Cells in Serum-free Medium Supplemented with SHITE. In many experiments, the population doubling times during both the logarithmic and high-density phase of growth were very similar in medium supplemented with 10% FCS or SHITE. However, the cells in serum-containing medium nearly always replicated at a slightly faster population doubling time and to a total cell density 1.5 to 4 times higher than in the hormone-supplemented medium. Therefore, another series of additions to the SHITE-supplemented medium was made and compared to growth in 10% FCS-supplemented medium (Table 4; Chart 2). FCS (0.1%) and bovine serum albumin (0.5%) had a slight effect on the low- and high-density phases, respectively. Addition of small concentrations of FCS, bovine serum albumin, fetuin, Pitressin, NGF, or combinations of NGF:putrescine, Pitressin:TRH, or cocktails of multiple hormones did not add to the growth of SHITE-stimulating activity in RPMI Medium 1640. However, the combination NGF:putrescine:TRH may have had a modest effect in RPMI Medium 1640 compared to SHITE particularly when the cell concentration was over 1×10^6 cells/well (Chart 3).

Testing for Optimal Concentrations of the Components of SHITE. While the other components of SHITE were held constant, one component at a time was varied over a 100-fold concentration range on either side of the concentration used in the standard formula (Chart 4). All of the components were at their optimal concentrations in the standard SHITE mixture, and increasing all of them resulted in some inhibition of growth. With the exception of transferrin, the absolute deletion of any of the individual components did not have a dramatic effect on the growth curve results. Deletion of insulin yielded cells which had a delay in entering the log phase of growth but then gave a similar population doubling time to that of the full combination. NGF was added to SHITE-supplemented RPMI Medium 1640 at several concentrations and was found to be inhibitory at concentrations above 10 ng/ml (Chart 4*E*).

Other Small Cell Carcinoma Lines Can Replicate in SHITE-supplemented Medium. We have developed a series of other lines from patients with small cell carcinoma of the lung that have been shown to be tumorigenic and exhibit APUD characteristics (17). These were tested for their ability to replicate in unsupplemented RPMI Medium 1640 and medium supplemented with 10% FCS or SHITE. All of the lines were found to replicate in SHITE-supplemented medium and in some cases as efficiently or more efficiently than in serum-supplemented medium (Table 5).

Long-Term Replication of NCI-H69 Cells and Maintenance of APUD Characteristics in Serum-free SHITE-supplemented Medium. NCI-H69 cells have been grown in SHITE-supplemented RPMI Medium 1640 without FCS for periods over 12 months. Their morphology and ultrastructure in serum-free medium with growth factors are similar to those of cells in serum-containing medium. The cells grow as suspension cultures and form clusters and chains, a phenomenon characteristic of the cells in serum-containing medium. Electron microscopic studies reveal many neurosecretory granules in the serum-free hormone-supplemented medium. The cells continue to stain for fluorogenic amine production with formaldehyde-induced fluorescence and exhibited high levels of L-dopa decarboxylase (EC 4.1.1.28) activity (250 nmol $^{14}CO_2$ released per mg protein per hr). When injected into athymic nude mice,

Table 4

Effect of additions of growth factors to RPMI Medium 1640 supplemented with SHITE on the growth of NCI-H69 cells

Addition	Relative cell no.[a]	
	Low density (Days 9–11)	High density (Days 23–25)
FCS (10%) to RPMI Medium 1640	1.3	1.8
SHITE	1.0 (78×10^4)[b]	1.0 (2.34×10^6)
SHITE +		
FCS (0.1%)	1.3	1.0
FCS (1%)	1.1	1.20
Bovine serum albumin (0.5%)	0.94	1.24
Fetuin (500 μg/ml)	1.0	0.97
Fetuin (1000 μg/ml)	1.3	0.85
Pitressin (10 ng/ml)	0.88	0.96
NGF (1 ng/ml)	1.1	0.88
NGF (10 ng/ml)	1.0	0.92
NGF (10 ng/ml) + putrescine (10^{-4} M)	1.1	1.0
Pitressin (10 ng/ml) + TRH (10^{-9} M)	1.0	0.99
NGF (10 ng/ml) + putrescine (10^{-4} M) + TRH (10^{-9} M)	1.3	1.40
Multiple hormones[c]	0.86	1.06

[a] Ratio of numbers of cells in RPMI Medium 1640:SHITE plus the indicated factor:number of cells in RPMI Medium 1640 supplemented with SHITE alone. On Day 0, 5×10^4 cells were plated.

[b] Numbers in parentheses, numbers of cells in RPMI Medium 1640 supplemented with selenium (3×10^{-8} M), insulin (10 μg/ml), hydrocortisone (10^{-6} M), transferrin (100 μg/ml), and 17β-estradiol (10^{-6} M).

[c] RPMI Medium 1640 supplemented with SHITE and ACTH (5 μU/ml), blood meal (10 μg/ml), calcitonin (0.1 mg/ml), Decadron (4×10^{-8} M), follicle-stimulating hormone (0.1 units/ml), somatostatin (10 ng/ml), glucagon (500 ng/ml), Gly-His-Lys (200 ng/ml), growth hormone (5 ng/ml), luteinizing hormone (500 ng/ml), NGF (10 ng/ml), prolactin (10 ng/ml), PTH (0.5 ng/ml), putrescine (10^{-4} M), thyrotropin (5 ng/ml), TRH (10^{-9} M), testosterone (10^{-8} M), Pitressin (10 ng/ml), T3 (3×10^{-10} M), fibroblast growth factor (50 ng/ml), and multiplication-stimulating activity (50 ng/ml).

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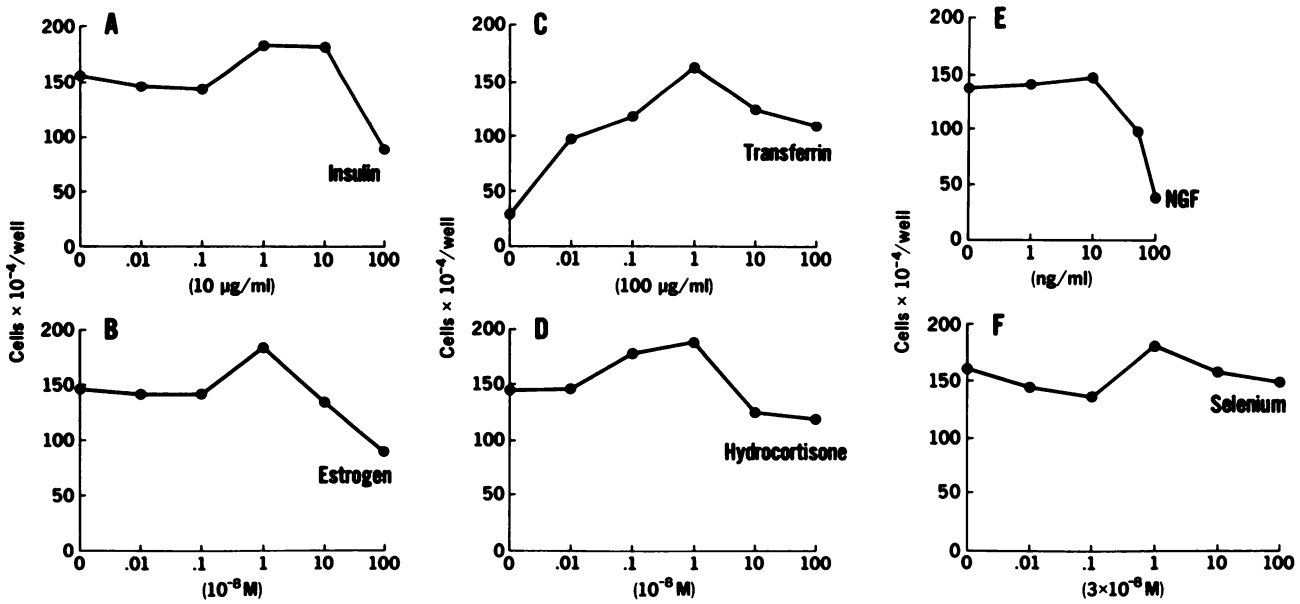

Chart 4. NCI-H69 cell numbers at Day 15 after 5 × 10^4 were plated per well in RPMI Medium 1640 supplemented with SHITE with various modifications of the individual hormone concentrations while the other components of SHITE were held constant. *A*, effect of varying insulin concentration (1 fold = 10 μg/ml); *B*, effect of varying the 17β-estradiol concentration (1 × 10^{-8} M); *C*, effect of varying the transferrin concentration (1 fold = 100 μg/ml); *D*, effect of varying the hydrocortisone concentration (1 fold = 10^{-8} M); *E*, effect of adding NGF at various concentrations to SHITE-supplemented medium; *F*, effect of varying the selenium concentration (1 fold = 3 × 10^{-8} M).

Table 5

Replication of various tumorigenic lines from patients with small cell carcinoma of the lung in RPMI Medium 1640 supplemented with SHITE or FCS

Human small cell carcinoma line[a]	Relative cell no. in additive:RPMI Medium 1640 alone		Day 16	No. of cells in RPMI Medium 1640 alone
	10% FCS	SHITE		
NCI-H64	52	21		2 × 10^4
NCI-H69	19	9.9		13 × 10^4
NCI-H128	6.1	9.5		19 × 10^4
NCI-N179	19.2	7.8		6 × 10^4
NCI-H60	19	7.0		5 × 10^4
NCI-N231	2.0	4.2		31 × 10^4
NCI-N230	3.2	2.9		29 × 10^4

[a] On Day 0, 10 × 10^4 cells were plated.

Table 6

Effect of deleting various growth factors from RPMI Medium 1640:SHITE on NCI-H69 cells grown in SHITE for 6 months

Growth factors	Relative cell no.[a] (Day 28)	Continuous growth after passage
SHITE	1.0 (4 × 10^6)	Yes
Minus hydrocortisone	1.2	Yes
Minus 17β-estradiol	1.1	Yes
Minus selenium	0.86	No
Minus selenium and 17β-estradiol	0.64	No
Minus insulin	0.27	No
Minus transferrin	0.04	No
Minus all	0.45	No
Minus hydrocortisone and 17β-estradiol	ND[b]	Yes

[a] Ratio of number of cells in text medium:number of cells in SHITE-supplemented medium. On Day 0, 5 × 10^4 cells were plated, average of triplicate values.

[b] ND, not determined.

they induced tumors (3/3 mice tested) with characteristic morphology of small cell carcinoma. The NCI-H69 cells grown for 6 months in SHITE were cloned in 0.25% agarose in RPMI Medium 1640 supplemented with either SHITE or 10% FCS. The colony-forming efficiency was 1.25% in 10% FCS and 1.2% in SHITE-supplemented medium, respectively.

Effects of Factor Deletions on NCI-H69 Cells Maintained in Serum-free SHITE-supplemented Medium for 6 Months. NCI-H69 cells passaged and maintained continuously in serum-free SHITE-supplemented RPMI Medium 1640 for over 6 months were tested for their dependence on the individual factors in SHITE by deletion of components (Table 6). Deletion of transferrin, insulin, or selenium eventually resulted in cessation of growth of the cell line. However, deletion of hydrocortisone, estradiol, or both together still allowed continued cell replication. When testing for cell number 3 or 4 weeks after factor deletion, the effects of deleting transferrin and insulin were the most dramatic, and deletion of all components simultaneously appeared to have a temporary protective effect compared to just deleting transferrin or insulin individually (Table 6). The deletion of hydrocortisone or estradiol did not affect the cell number at 28 days. The cells in the various deleted media were passaged after 28 days and subsequently maintained in the deleted media, and the combination selenium:insulin:transferrin appeared to be an absolute requirement for continued growth.

DISCUSSION

A tumorigenic human cell line (NCI-H69) which expresses a variety of histological and biochemical characteristics of small cell carcinoma of the lung was tested for its ability to replicate in serum-free medium and medium supplemented with various growth factors and hormones. The results show that RPMI Medium 1640 or F14 medium supplemented with the combination selenium (3 × 10^{-8} M):hydrocortisone (10^{-8} M):insulin (5 μg/ml):transferrin (5 to 100 μg/ml):17β-estradiol (10^{-8} M) (SHITE) is sufficient to allow continued replication of NCI-H69 cells for periods of over 12 months. After a slight delay, the

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population doubling time in the logarithmic phase of growth is similar to that of FCS-supplemented medium, but the saturation densities reached are slightly lower. While addition of many individual hormones and growth factors to this hormone combination did not significantly alter the log or high-density growth pattern, the addition of a combination of NGF:putrescine:TRH appeared to have a modest effect in media based on RPMI Medium 1640 in increasing cell number particularly under high-density growth conditions. Addition to RPMI Medium 1640: SHITE of NGF, NGF:putrescine, or Pitressin:TRH alone did not have a stimulatory effect on cell number, and putrescine:NGF was inhibitory when added to F14:SHITE. Also, the presence of multiple other hormones inhibited the RPMI Medium 1640: SHITE:NGF:TRH:putrescine effect. In any event, continued application for over 6 months took place in RPMI Medium 1640:SHITE.

We found that a series of 6 other small cell carcinomas of the lung cell lines was also able to replicate in RPMI Medium 1640:SHITE indicating the generality of the formula for this histological type of lung cancer. In addition, NCI-H69 cells grown in serum-free SHITE-supplemented medium for long periods of time retained their histology, tumorigenicity, agarose clonogenicity, and biochemical properties of APUD-derived small cell carcinomas.

After 6 months of growth in RPMI Medium 1640:SHITE, only insulin, transferrin, and selenium appeared to be stringently required for continued growth. The ability of these cells to replicate continuously in serum-free media without the steroid hormones (hydrocortisone and estradiol) suggests that these hormones may be stimulatory on initial transfer from serum-containing medium but not necessary for long-term growth or that some cells may be turned on to produce one or the other of these compounds when not supplied.

We arrived at the SHITE formula by adding various hormones and growth factors to the combination IT in serum-free medium. Insulin and transferrin are requirements in the other reported defined media (5). When insulin or transferrin was deleted from the final SHITE formula, significant reduction in NCI-H69 replication was noted in the low-density phase of growth with the deletion of transferrin being the most dramatic in both the low- and high-density growth phases. We found that the concentrations of selenium, hydrocortisone, and estradiol were optimal for NCI-H69 replication but that deletion of any one of these components from SHITE did not dramatically alter the growth of these cells in serum-free medium over a 24-day observation period. However, the combination IT alone did not yield optimal growth compared to the full SHITE combination. Whether other factors could be substituted for hydrocortisone and estrogen remains to be determined.

Patients with small cell carcinoma of the lung are known to have elevated levels of various hormones presumably related to production by tumor cells (27). These frequently include ACTH (and thus possibly the endorphin, β-lipotropin:enkephalin complex), AVP, calcitonin, neurophysin, and oxytocin. Infrequently, a variety of other hormones have been reported to be elevated in these patients (27). Also, there are reports of *in vitro* cell culture lung cancer lines producing ACTH:endorphin, AVP, calcitonin, estrogen, and other hormones (1, 3, 4, 9, 14, 15, 18, 26, 30). In fact, AVP is mitogenic for mouse 3T3 cells (28). Thus, it is possible that the small cell carcinoma cell cultures may produce some of the hormones or growth factors required for their replication in serum-free or serum-supplemented medium. If these factors have growth-stimulatory effects *in vivo*, then tumors in patients may be producing factors which could stimulate their own growth.

Current evidence suggests that small cell carcinomas of the lung are derived from part of the APUD cell series (24, 31). The embryonic derivation of this diffuse neuroendocrine system and the relation to neuroepithelial cells are the subjects of investigation (31). At present, over 40 cell types make up this system (25). Other cell lines putatively related to the APUD series and thus to small cell carcinoma of the lung have had their serum-free growth factor requirements determined (5–8). Bottenstein *et al.* (5) and Bottenstein and Sato (6) found that the combination insulin (5 μg/ml):transferrin (5 or 100 μg/ml): selenium (3×10^{-8} M):progesterone (2×10^{-8} M): putrescine (10^{-4} M) will support the replication of B104 rat neuroblastoma cells, PC12 rat pheochromocytoma cells, and N1E-115 mouse adrenergic neuroblastoma cells. By adding NGF to this combination, Bottenstein and Sato (6) found they could selectively maintain chick dorsal root ganglion neurons in an undividing state in the serum-free supplemented medium.

Bottenstein and Sato (6) found that each supplement added individually had little effect but that in combination, there was a synergistic effect on cell growth. Bottenstein and Sato (6) found an inhibitory effect of substituting β-estradiol (2×10^{-8} M) or testosterone (2×12^{-8} M) for progesterone in their formula. We found no effect of adding progesterone (10^{-8} M) or putrescine (10^{-4} M) in our initial tests, and in modified F12 medium (F14 medium), putrescine was inhibitory. Thus, there appears to be a different growth response between small cell carcinomas and neuroepithelial lines to the defined media combinations.

Bottenstein *et al.* (7), Bottenstein and Sato (6), and Honneger *et al.* (21) reported continued expression of neuroepithelial function in serum-free hormone-supplemented media. Likewise, the small cell carcinoma lines continued to express characteristic histology, neurosecretory granules, formaldehyde-induced fluorescence, and L-dopa decarboxylase activity. Thus, these defined media should be of use in studying the regulation of differentiated functions in these cell types. In particular, this medium should allow the study of hormones produced by small cell carcinomas.

Some of the hormones and growth factors when added to serum-free supplemented media at low concentrations inhibited the growth of NCI-H69 cells. This was noted with blood meal extract, EGF, Gly–His–Lys, LHRF, and PTH. Whether these factors generally will inhibit the growth of small cell carcinoma remains to be determined. The identification of such growth regulators may have therapeutic implications.

Finally, in an extensive study of the cell culture of small cell carcinoma, we have repeatedly noted that while this tumor grows very well in patients, when put into serum-containing medium, it is extremely difficult to derive continuously replicating cell lines unless conditioned medium is used (17). In addition, while such tumor cells will initially clone in semisolid serum-containing medium, this is at a low efficiency, and such clones when picked do not yield continuously replicating cell lines (11). Thus, it appears that some other growth factors are required for small cell replication or that FCS contains inhibitors for small cell carcinoma replication. The identification of a defined medium which readily allows the *in vitro* replication,

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cloning, and expression of APUD characteristics of small cell carcinoma lines should have application for studying the biology of this and other cell types of the APUD series, allow the identification of factors which can regulate the growth of these tumor cells, and provide a new selective system to isolate tumor cells from patient specimens free of other cell types in which to test chemotherapeutic agents.

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