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Isolation of tumor-secreted products from human carcinoma cells maintained in a defined protein-free medium

(serum-free culture medium/conditioned medium/tumor antigens/growth factors/glutamine)

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ABSTRACT **A protein-free synthetic cell-growth medium has been defined that permits long-term survival (>120 days) of an established human colon tumor cell line, HT-29. Viability is dependent upon both the concentration of L-glutamine in the medium and the cell density at the time of initial transfer into it. Cell proliferation is minimal, thus obviating the necessity for subculturing. HT-29 adenocarcinoma cells maintained in large-scale culture with this medium continue to secrete the established colon tumor marker carcinoembryonic antigen as well as growth factors and lysozyme. These and, potentially, other important tumor-derived products can therefore be generated continuously in such cultures so that they can be isolated from a conditioned medium free of contaminating serum and protein supplements.**

The large-scale *in vitro* cultivation of malignant cells can potentially produce tumor-derived biomolecules in yields sufficient for both biological and chemical characterization. However, for this purpose exogenous serum and/or its growth factor components[§] are virtually compulsory additives. This has greatly complicated the procedure itself and, beyond that, the very objective, i.e., the identification and purification of tumor constituents which are usually present in vanishingly small amounts. Successful attempts to culture tumor cells under serum-free conditions have been reported, but, while eliminating addition of serum, they have substituted moieties obtained from it (1–3). Moreover, each cell line generally then requires a set of such molecular species unique for its survival and devised to bring about cellular proliferation rather than maintenance of long-term viability and secretory capacity. The latter has proven difficult if not impossible to achieve in this manner.

The present studies, part of our effort to isolate and characterize tumor-secreted products, were aimed at maintaining long-term viability rather than proliferation of human tumor cells in serum-free media and in the absence of serum-generated factors. The medium that we have developed is devoid of exogenous proteins or other growth factors and supports the survival of at least two colon adenocarcinoma lines which remain physiologically active with respect to some functions.

MATERIALS AND METHODS

Cell Lines. All tumor cells were of human origin and included the colorectal adenocarcinoma lines HT-29 (4), WiDr [ATCC (American Type Culture Collection) CCL 218], COLO 201 (ATCC CCL 224), and COLO 205 (ATCC CCL 222); the lung carcinoma line A549 (ATCC CCL 185); and the fibrosarcoma line HT-1080 (ATCC CCL 121). Cells were propagated routinely in T-flasks (Costar, Cambridge, MA) as monolayer cultures in Dulbecco's modified Eagle's medium (M. A. Bioproducts, Walkersville, MD) containing glucose (4.5 mg/ml), gentamicin (50 μg/ml), and Fungizone (0.5 μg/ml) (DME) and supplemented with 5% heat-inactivated fetal bovine serum and 2 mM L-glutamine (DME/5%). BALB/c 3T3 cells, clone A31-MK25, were provided by M. Klagsbrun and were maintained in DME supplemented with 2 mM L-glutamine and 10% heat-inactivated bovine serum. Cultures were incubated at 37°C in humidified air maintained at 7% CO_2. Cells were fed every 2–3 days as required and subcultured at confluence by standard trypsinization techniques.

Cell Growth Curves. To evaluate HT-29 growth in serum-free medium, cells maintained as above were trypsinized, resuspended in DME/5%, and seeded into 24-well (1.9-cm^2) Nunc microplates (Vangard International, Neptune, NJ). At specified intervals, conditioned medium was removed and adherent cells were harvested with trypsin. Cells in duplicate wells were counted microscopically after vital staining with acridine orange/ethidium bromide and average values were calculated. In some instances, conditioned medium was harvested and nonadherent cells were counted as above. Remaining cultures were refed with 1 ml of protein-free medium as described.

Protein-free maintenance of other cell lines was evaluated by plating cells in 100 μl of DME/5% into 96-well, half-area (0.015-cm^2) microplates (Costar). At levels approaching confluence ($\approx 1.2 \times 10^5$ cells per cm^2), cultures were transferred into protein-free medium and refed as indicated. Adherent cells were counted using an Artek model 982 image analyzer interfaced with an Apple II+ computer. Twelve wells were counted per data point and the average of these counts was calculated.

Large-Scale, Protein-Free Cultures. HT-29 cells, maintained as above, were seeded into a 6000-cm^2 Nunc multilevel "cell factory" (Vangard International), using an inoculum of 1×10^8 cells in 1.5 liters of DME/5%. At 80–90% confluence (3–5 days later) the standard growth medium was replaced with 1.5 liters of protein-free maintenance medium. Conditioned medium was harvested at 2- to 3-day intervals and clarified by sequential passage through Whatman 40 filter paper and Whatman 934-AH glass microfiber filters. The resulting serum-free conditioned medium (SFCM) was frozen and stored at −20°C for subsequent evaluation. Cells in cell

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Abbreviations: DME, Dulbecco's modified Eagle's medium supplemented with glucose (4.5 mg/ml), gentamicin (50 μg/ml), and Fungizone (0.5 μg/ml); DME/5%, DME supplemented with 5% heat-inactivated fetal bovine serum and 2 mM L-glutamine; SFCM, serum-free conditioned medium; CEA, carcinoembryonic antigen.

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§It is understood, of course, that serum supplies growth factors, hormones, transport and attachment factors, or lipids whose nature, amounts, or mechanisms of action remain largely unspecified.

factories were refed after each harvest with 1.5 liters of freshly prepared protein-free medium.

Evaluation of SFCM for Extracellular Products. Bovine serum albumin and carcinoembryonic antigen (CEA) were quantitated by the rocket immunoelectrophoretic method of Laurell (5) using the appropriate rabbit antiserum (Accurate Chemicals, Westbury, NY). The CEA used as a standard was obtained from Calbiochem–Behring (San Diego, CA). Lysozyme activity was determined spectrophotometrically according to the turbidometric method of Locquet *et al.* (6) as described (7). Growth factor activity was determined by stimulation of quiescent confluent monolayers of 3T3 cells as described (8). Prior to the growth factor and CEA assays, SFCM was dialyzed versus water using 6000–8000 molecular weight-cutoff tubing (Spectra/Por) and concentrated 100-fold by lyophilization and reconstitution.

RESULTS

Development of Protein-Free Maintenance Medium. The overall strategy was first to define serum-free conditions in which cells would remain viable for relatively long periods of time (>20 days) and then to assess their secretory capabilities under such conditions. To establish basal conditions from which to proceed, 1-ml cultures each containing 10^5 HT-29 cells were seeded into 24-well microplates in DME/5% and allowed to grow to 80–90% confluence (13–15 × 10^5 cells per well). Medium was then replaced and cultures subsequently were refed twice weekly with DME (minus serum) supplemented with the standard addition of 2 mM L-glutamine. Adherent cells were harvested and counted in duplicate wells at the time of refeeding. The results of this initial experiment are depicted in Fig. 1. Remarkably, HT-29 cells survived in the absence of serum or any added growth factors for the entire 21-day course of the experiment. Further, the number of viable adherent cells remained essentially constant throughout the experiment, suggesting that, for this population, cell proliferation was minimal under the conditions defined (see below).

In this initial experiment, L-glutamine was the only supplement to DME. Since its importance in mammalian cell biology is well documented (see *Discussion*), its apparent requirement in this setting called for more detailed examination. Hence, protein-free medium was supplemented with several concentrations of L-glutamine to discern its effect on cell growth and survival. The data (Table 1) depicting adherent cell counts on days 3, 7, and 31 show that addition to DME of 5 mM L-glutamine provides an advantage over the addition of 2 mM L-glutamine for supporting the survival of the greatest number of viable cells over the 31-day period. There was no significant advantage gained by the addition of greater quantities of L-glutamine up to 15 mM. This was subsequently found to be independent of the cell concentration at the time of transfer into protein-free, L-glutamine-supplemented DME (data not shown). In no instance did the percentage of adherent cells that were nonviable exceed 5%. Moreover, when nonadherent cells were similarly counted in cultures maintained in DME/5 mM glutamine, they were found to amount to about 1% of the total adherent population and to be more than 90% viable (data not shown). The nonadherent population remains essentially constant throughout the course of the experiment just as the adherent population. These findings indicate that when HT-29 cells are transferred into DME/5 mM glutamine, their proliferation is minimal and a dynamic cellular proliferation balanced by cell death does not occur.

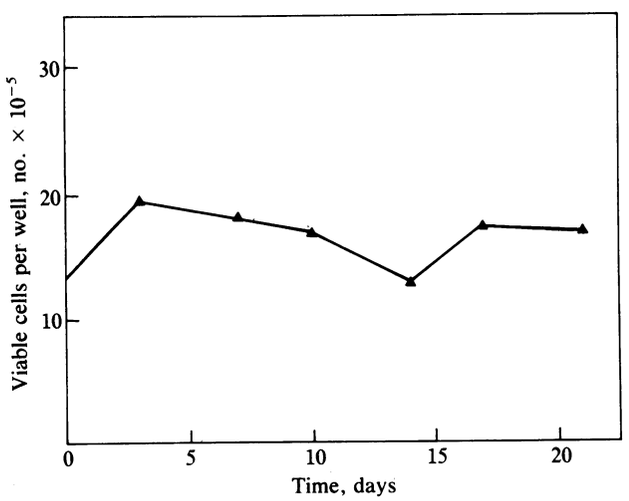

FIG. 1. Growth curve of HT-29 cells in protein-free medium. Cells were plated at 10^5 cells per 1.9-cm^2 well in 1 ml of DME/5% and grown to a density of 13.5 × 10^5 cells per well. Subsequently (day 0), the medium was replaced with 1 ml of DME supplemented with 2 mM L-glutamine. Adherent cells were harvested and counted on the days indicated, and cells in remaining wells were refed with protein-free medium. Data are averages of duplicate wells. Nonviable cells did not exceed 6% of the total adherent cell population.

To examine whether there is an initial selection for a competent subpopulation of adherent cells upon transfer into DME/5 mM glutamine, the experiment depicted in Fig. 2 was performed. HT-29 cells were plated as above in DME/5% and grown to a density of 6 × 10^5 cells per well. Cultures were refed at this time with 1 ml of DME/5 mM glutamine, and adherent cells were counted 7, 24, 56, and 75 hr later. There was no significant initial loss of adherent cells. In fact, after a short lag period, cells continue to divide until the culture approaches confluence and a growth plateau is reached.

Density Dependence of Survival in DME/5 mM Glutamine. Cells were plated at 10^5, 5 × 10^4, 10^4, and 5 × 10^3 cells per 1.9-cm^2 well in 1 ml of DME/5% and allowed to grow for 72 hr. At this time (day 0), medium was replaced with 1 ml of DME/5 mM glutamine. As above, adherent cells were counted and medium was replaced in remaining wells twice weekly. Fig. 3 shows that a critical population density of HT-29 cells at the time of transfer into protein-free maintenance medium is required to sustain survival beyond 18 days. At a threshold level of ≈2 × 10^4 cells per cm^2, HT-29 cells can be maintained, but below this level long-term survival cannot be achieved. This is in contrast to culture of HT-29 cells in DME/5%, where clonal growth is observed (data not shown).

Large-Scale Cultivation and Evaluation of Extracellular Products. After a maintenance medium for HT-29 cells in protein-free conditions had been defined, large-scale cultivation was initiated to provide sufficient amounts of condi-

Table 1. Growth of HT-29 cells in DME supplemented with various concentrations of L-glutamine

Glutamine supplement, mM	Viable cells per well, no. × 10^{-6}		
	Day 3	Day 7	Day 31
2	0.8	2.0	1.7
5	0.8	2.2	2.8
7	0.7	1.2	2.1
10	0.7	1.6	2.1
15	0.5	1.4	1.3

Cells were seeded at 10^5 per 1.9-cm^2 well and grown to 14.5 × 10^5 cells per well. At this time (day 0), medium was replaced with DME (1 ml per well) supplemented with various amounts of glutamine. Medium was subsequently replaced twice weekly. Data presented are viable adherent cell counts (average of duplicate wells).

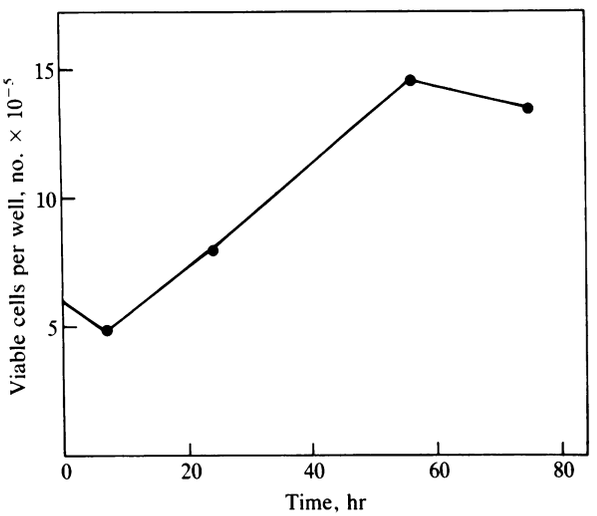

FIG. 2. Short-term growth curve of HT-29 cells in protein-free medium. Cells were plated at 10^5 cells per 1.9-cm^2 well in 1 ml of DME/5% and grown to a density of 6×10^5. Subsequently (day 0), the medium was replaced with 1 ml of DME/5 mM glutamine. Medium was not replaced again during the experiment. Adherent cells were harvested and counted at the indicated times; data are presented as the average of duplicate wells. Nonviable cells did not exceed 5% of the total adherent cell population.

tioned medium to examine extracellular products. Cells (10^8) were seeded into a 6000-cm^2 multilevel "cell factory" with 1.5 liters of DME/5%. After 3–5 days in culture, the standard growth medium was replaced with 1.5 liters of DME/5 mM glutamine. Harvesting and initial processing of conditioned medium and refeeding were described under *Materials and Methods*. HT-29 cells synthesize and secrete large amounts of lysozyme (7), which provides a convenient marker of cellular secretory capacity. Growth factor activity for 3T3

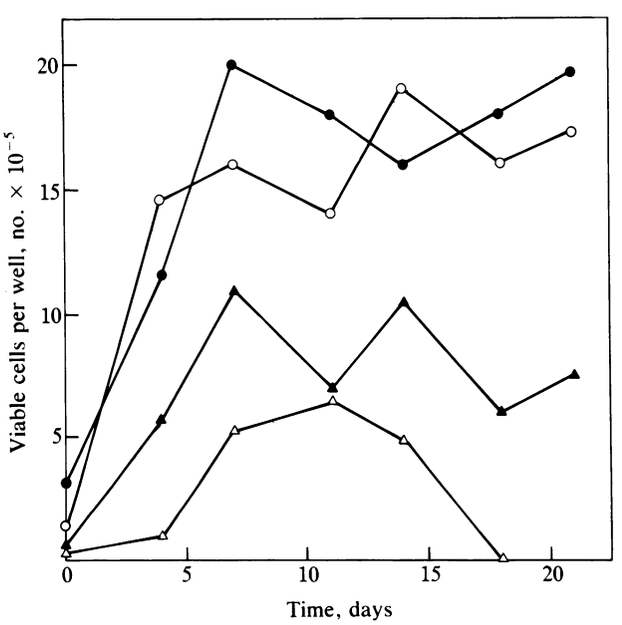

FIG. 3. Density-dependent growth of HT-29 cells in protein-free medium. Cells were plated in 1 ml of DME/5% at 10^5 (●), 5×10^4 (○), 10^4 (▲), or 5×10^3 cells per well (△). After 72 hr of growth (day 0), medium was replaced with 1 ml of DME/5 mM glutamine. Adherent cells were harvested and counted on the days indicated, and cells in remaining wells were refed with protein-free medium. Data are presented as the average of duplicate wells. Nonviable cells did not exceed 6% of the total adherent cell population.

Table 2. Release of lysozyme, CEA, and growth factor(s) during protein-free maintenance of HT-29 cells

Day	Lysozyme, ng/ml	CEA, ng/ml	3T3 growth-promoting activity, units/ml
0*	352	77	ND†
2	300	63	ND†
7	435	70	0.55
14	381	52	0.37
21	182	35	0.30
28	154	35	0.28

Data are average values obtained from two separate cell factories.
*Conditioned medium containing fetal bovine serum was harvested before the cells were transferred into DME/5 mM glutamine.
†Not determined, because of the expected presence of serum-derived growth factors (see text).

cells as well as CEA concentrations were also measured in the SFCM. The cells in two factories were maintained for 4 weeks and the average lysozyme, growth factor, and CEA levels were determined throughout this period (Table 2). There appears to be no initial loss of lysozyme-secretory capacity when cells are placed in serum-free medium, although values do tend to decrease with time. This was determined by comparing the lysozyme value at day 0—i.e., in conditioned medium harvested before transfer into DME/5 mM glutamine—with those obtained for subsequent harvests. Conditioned medium obtained at days 7, 14, 21, and 28 also contains growth factor activity. Medium harvested at day 0 and at day 2 was not examined for this activity since fetal bovine serum and, hence, serum-derived growth factors were still expected to be present (9). By day 7, however, serum-derived albumin could not be detected immunochemically in the SFCM (limit of detection ≈200 ng/ml). Additionally, SFCM contains immunochemically detectable amounts of the HT-29 product CEA (10). As in the case of lysozyme, growth factor activity and CEA levels decrease with time. Thus, HT-29 cells maintained under the specified protein-free conditions not only survive for long periods but also retain their capacity to secrete tumor-derived products.

We have been able to maintain individual factories in DME/5 mM glutamine for more than 120 days. When losses have occurred, they have been due to contamination or cell detachment rather than observable loss of cell viability. However, as shown in Table 2, the yield of extracellular, tumor-derived products does decrease with time and, as a consequence, factory cultures are routinely replaced after 60 days.

Protein-Free Culture of Other Cell Lines. In addition to the HT-29 cells, three other colon carcinoma lines (WiDr, COLO 201, and COLO 205), one lung carcinoma line (A549), and one fibrosarcoma line (HT-1080) were studied to determine their ability to survive under the protein-free conditions developed for HT-29. Of these, one cell line, WiDr, can be maintained in long-term culture (>20 days) when transferred at high density into DME/5 mM glutamine (Fig. 4). Moreover, the SFCM harvested from WiDr cultures at day 21 contains immunochemically detectable amounts of CEA (data not shown).

DISCUSSION

Tumor-secreted products play critical roles in malignant growth. They subserve a multitude of functions including interference with the host immune response (11, 12), autostimulation of tumor cell growth (13, 14), modulation of invasion and metastasis (15), and induction of neovascularization (16, 17). The chemical and biological characterization of such molecules continue to provide valuable insight into the mechanisms governing the complex biological processes

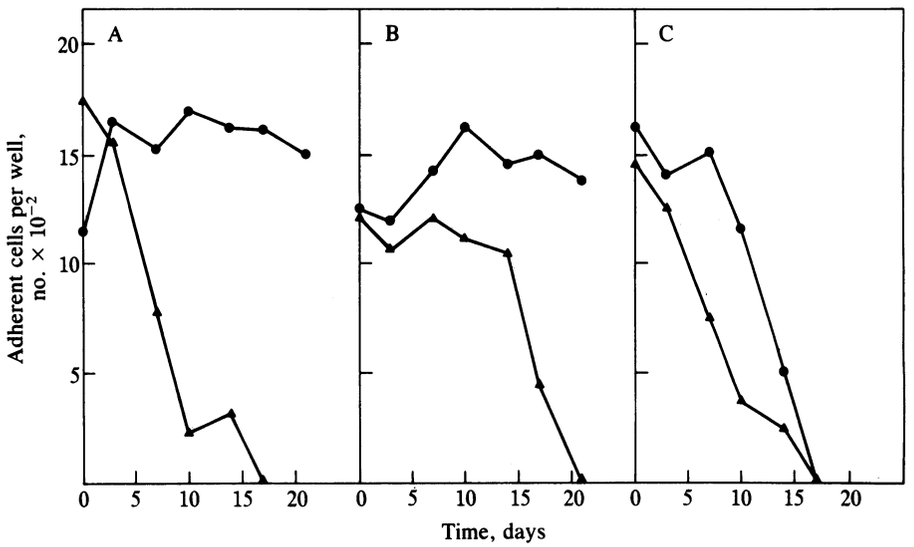

FIG. 4. Comparison of growth for several cell lines in protein-free medium. Cells were plated at 5×10^2 cells per 0.015-cm^2 well in 100 μl of DME/5%. After 72 hr of growth (day 0), medium was replaced with 100 μl of DME/5 mM glutamine. At various times adherent cells were counted with an Artek image analyzer and remaining cells were refed with protein-free medium. Each data point represents the average of 12 wells counted. (*A*) HT-29 (●) and A549 (▲). (*B*) WiDr (●) and COLO 205 (▲). (*C*) COLO 201 (●) and HT-1080 (▲).

of malignancy while also providing new means for cancer detection, diagnosis, and therapeutic management. The identification as well as the structural and functional characterization of such molecules depends critically on the availability of systems from which such molecules can be isolated in high yield. Although *in vitro* cultivation of established tumor lines remains the method of choice, it is laborious to purify tumor-secreted products to homogeneity from such cultures. This is due, in part, to the typical serum requirements of most cells, since its presence complicates the purification of such products.

Techniques for cultivating both primary and established lines of mammalian cells in defined, serum-free but supplemented medium are now well developed. Such media can provide a suitable environment for cell proliferation but, with few exceptions (18–21), require supplementation with various hormones and proteins (1). Further, each new cell type usually requires a unique, defined medium for serum-free growth. The nutrient requirements of quiescent but actively metabolizing cells, however, are much less than those of proliferating cells (3). During studies of tumor-secreted products, including tumor angiogenesis factor (17), we investigated the possibility of keeping established human tumor lines in a serum-free medium optimized for cell survival and the maintenance of secretory capacity. A medium that supports cell viability but not proliferation should permit long-term harvesting of secreted products without the need for extensive manipulation and subculturing of cells. It would obviate the isolation of products synthesized in very low yield from large amounts of protein added to the medium for growth.

The protein-free maintenance medium defined here supports the long-term survival of at least two established human colon tumor cell lines, HT-29 and WiDr. The medium consists of the basal nutrient mixture of DME (containing antibiotics) supplemented only with 5 mM L-glutamine. Glutamine, therefore, appears to enhance survival of these cells under the conditions employed.

Glutamine occupies a pivotal position in numerous biochemical pathways and seems to play a unique role as a substrate in oxidative cellular metabolism (22, 23). It is used as a major energy source by both kidney and intestinal tissues *in vivo* (24–26). The latter observation is relevant to the observed survival capacity of established colon carcinoma lines in our glutamine-supplemented medium. Other studies provide evidence that glutamine serves as a major energy source not only for normal cells *in vivo* but also for tumor cells cultivated *in vitro*. Energy provision through glutamine utilization has been described for several tumor types which include HeLa, lymphoma, and myeloma cells (27–29). Moreover, increased glutamine utilization has been shown to correlate with malignant growth *in vivo* (30, 31). Thus, the major cellular changes that accompany malignant transformation may involve, in part, a switch to glutamine as the major energy substrate (32, 33). It is not surprising, in light of the key role that this amino acid serves in the metabolism of normal and malignant mammalian tissues both *in vivo* and *in vitro*, to find that glutamine supplementation aids in the long-term survival of tumor cell lines. However, it is still unknown what function(s) glutamine serves in the maintenance of survival of these cells.

Some of the limiting conditions for the maintenance medium have also been defined. For the HT-29 line, cell density at the time of shift to maintenance medium is a critical consideration for survival. This observation, which has been reported in other serum-free systems (20, 21, 34), strongly suggests that nutrients and/or hormone-like substances produced by the tumor cells themselves are required for cell survival even in the presence of added glutamine. Such growth-promoting substances have been described and isolated from animal and human tumor sources (34, 35), and potent growth factor activity has been found in the HT-29 serum-free conditioned medium (Table 2). Whether the factor(s) we detected is autostimulatory, as has been demonstrated in other systems (13, 14), has yet to be determined. However, autologous conditioned medium is required for protein-free passage of HT-29 cells (unpublished data). Additionally, only anchorage-dependent tumor cells may be capable of long-term survival in our maintenance medium, since all attempts to maintain suspension-adapted HT-29 and WiDr cells in protein-free medium have been unsuccessful.

Although two established cell lines can be maintained in DME/5 mM glutamine under the conditions described, four other lines did not survive (Fig. 4). For such lines, parameters such as glutamine concentration in the protein-free medium, frequency of refeeding, composition of basal nutrient medium, and effects of hormone and protein supplementation, among others, should be examined. Nevertheless, our data do suggest that it may be possible to maintain yet other cell

lines if they are transferred at high density into DME/5 mM glutamine.

Large-scale, protein-free cultivation of HT-29 cells is currently under way. Five liters of conditioned medium per week can be harvested from each cell factory maintained as above. As stated, minimal harvesting and refeeding is required since subculturing is not necessary. To date, a number of secreted, tumor-derived products have been detected, including lysozyme, whose purification and characterization were described previously (7). Additionally, several other molecules have been identified and partially purified from protein-free conditioned medium of HT-29 cells. These include CEA, 3T3 cell growth factors, β_2-microglobulin, an angiogenic factor, and a vascular permeability factor. These will be the subjects of future reports.

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