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Establishment and Characterization of an *in Vitro* Model System for Human Adenocarcinoma of the Stomach[1]

S. C. Barranco,[2] C. M. Townsend, Jr., Cacilda Casartelli, B. G. Macik, N. L. Burger, W. R. Boerwinkle, and W. K. Gourley

Departments of Radiation Therapy [S. C. B., W. R. B., C. C., B. G. M., N. L. B.], Surgery [C. M. T.], and Pathology [W. K. G.], University of Texas Medical Branch, Galveston, Texas 77550

ABSTRACT

Ten permanent clones derived from a single biopsy specimen of an untreated human adenocarcinoma of the stomach were established and characterized *in vitro*. Tissue culture growth properties, doubling times, plating efficiencies, growth fractions, cell cycle phase distributions, DNA indices, modal chromosome numbers, and ploidies were determined. Growth fractions were nearly 100%, and doubling times ranged from 23 to 37 hr. The plating efficiencies were generally high for tumor cells in culture, ranging up to 70%. Modal chromosome numbers varied from 45 to 48, with a wider range of variability in about 25% of the cells studied in each clone. In addition, the parent cell line (from which the clones were isolated) was shown to grow in athymic mice and to have the same histochemical and cytological characteristics as the specimen taken from the patient.

It is important to characterize human tumor cells *in vitro* in this detailed manner, since they serve as excellent model systems for other studies involving the heterogeneous responses to drugs and radiation. The identification of mechanisms of drug sensitivity and resistance and the testing of drug and radiation combination treatment schedules in such *in vitro* systems can provide valuable insight into the design of clinical protocols for treatment of stomach cancer in humans.

INTRODUCTION

Although the absolute incidence of carcinoma of the stomach has declined over the last 30 years, carcinoma of the stomach still ranks seventh as the cause of cancer deaths in America (15); in 1981, there were 24,000 new cases and 14,000 deaths in this country (16). Surgical excision remains the only potentially curative treatment for cancer of the stomach. However, Moertel and Reitemeier (32) have noted that while 75 to 90% of patients with cancer of the stomach were candidates for laparotomy and 50 to 60% of patients had potentially curative resection, the overall 5-year survival rate for carcinoma of the stomach is between 5 and 15%. These rates have not changed dramatically in the last 30 years. In 3 large series of patients in whom the cancer was confined to the stomach (only 15% of all patients who were operated upon), 5-year survival was 57% (11, 23, 43). When regional lymph nodes were involved, 5-year survival was reduced to between 5 and 14.5%. In patients who have unresectable cancer of the stomach, the median duration of survival from diagnosis is 4 months (32).

Currently used chemotherapeutic regimens have produced little or no significant improvement in survival in patients with carcinoma of the stomach. This is possibly due to the presence of heterogeneous mixtures of multiclonal tumor cells (26, 33, 38, 51, 52). The multiclonal nature of tumor cells can be expressed as differences in nutritional requirements, variable responses to growth factors, and altered enzyme levels and accounts in part for differences in metastatic potential and heterogeneous responses to chemotherapy agents or radiation (3–6, 8, 10, 12, 13, 20, 22, 28, 34, 36, 37, 39, 40, 44–46, 48, 49). Recent *in vivo* evidence obtained by flow cytometry techniques indicates that some solid tumors are composed of 2 or more clones of tumor cells having widely different DNA contents (1, 10, 42, 44). Other evidence (42) confirms that some of these clones disappear or are greatly reduced following chemotherapy while the relative fractions of other clones within the same tumor are observed to increase during the same interval, suggesting resistance; these are the predominant tumor clones present at the death of the patient.

For patients who have gastric cancer that cannot be totally removed by surgical excision (regional lymph node metastases and unresectable disease), the prognosis remains dismal. These data emphasize the importance of the need for effective, systemic therapy for the vast majority of patients with cancer of the stomach. What has been lacking is a suitable *in vitro* human stomach cancer model system on which to test for the heterogeneous drug and radiation effects, cell cycle responses, and repair and recovery from drug- or radiation-induced damage and treatment schedules. We have now established an *in vitro* model system for human stomach cancer, and in this paper we report the *in vitro* characterization of 10 clones of tumor cells isolated from a single biopsy of an untreated human adenocarcinoma of the stomach. The responses of these clones to radiation, anticancer drugs, and other agents will be the subject of subsequent reports.

MATERIALS AND METHODS

Tumor Isolation and Cloning Techniques. A sterile segment of a freshly resected adenocarcinoma of the stomach was obtained, and the presence of viable tumor cells was confirmed by frozen section histological examination. The patient had received no prior cancer therapy. The tumor sample was transported from the operating room to the tissue culture laboratory in sterile Ham's F-10 medium with 20% FCS[3] (Grand Island Biological Co., Grand Island, N. Y.) and supplemented with high concentrations of antibiotics [penicillin (500 units/ml), streptomycin (300 μg/ml), and gentamicin sulfate (100 μg/ml); Schering Corp., Kenilworth, N. J.]. The tumor sample was separated from other associated tissues and washed in several changes of F-10 medium with antibiotics. The

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[2] To whom requests for reprints should be addressed.

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[3] The abbreviations used are: FCS, fetal calf serum; PE, plating efficiency; T_D, doubling time; FMF, flow microfluorometry; PAS, periodic acid-Schiff.

tumor was minced with scalpels and scissors in 1 ml of medium to pieces of 1 cu mm and smaller. Aliquots of the dissociated tumor (0.2 ml) were transferred to each of 5 plastic flasks containing 3 ml F-10 medium (with 20% FCS) and antibiotics. We used higher than normal concentrations of antibiotics to avoid the problem of contamination in gastrointestinal tumors. The cultures were incubated at 37° in a humidified incubator with 5% CO_2:95% air for 7 days. After this time, cultures were inspected daily and fed as needed with Ham's F-10 medium (20% FCS), and the antibiotics were reduced to standard concentrations [penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamicin (25 μg/ml)].

By the end of 30 days, there were numerous areas within each primary culture flask where apparently "pure" tumor clones were growing. These "pure" colony areas were in contrast to other places in the flasks where mixtures of fibroblasts, tumor, and other cells were growing together around small fragments of the original tumor sample. Several clonies from each flask were isolated using the following technique. The flasks were rinsed several times with 0.85% NaCl solution to remove floating and loosely attached cells. Sterile cloning rings (made from 6-mm glass tubing) were secured with sterile silicone grease around each selected colony. The silicone grease effectively isolated each colony, thus minimizing contamination with other cells. Each colony was removed from the cloning ring with trypsin (0.1% for 5 min) and was pooled with other colonies isolated in this manner into a secondary culture flask. This mixture of cells was designated the "parent culture" and was fed daily until it reached confluency (about 2 weeks). They were subcultured (1:2) every other day, and some cells were frozen (10% glycerol in medium) for future use.

After these cells had been in secondary culture for 90 days, they were cloned a second time. Known numbers of single cells (about 300 cells) of the parent culture were plated into 60-mm tissue culture dishes and allowed to grow into colonies for 2 weeks. Colonies were selected for cloning on the basis of differences in size and in cellular and colony morphologies. Twelve colonies were selected and designated AGS-1 through AGS-12; the parent culture is called AGS.

Cell and Culture Techniques. During the secondary culture stage (second to fourth months in culture), the cells were tested for growth in various media including McCoy's Medium 5A, CMRL 1066, F12, NCTC 135, Ham's F-10, Medium 199, Waymouth, and Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co.). The cells had the best overall growth properties in Ham's F-10 medium which is now used for culture maintenance and in experiments. All clonal and parent lines are maintained as monolayer stock cultures in exponential growth and are routinely checked for pleuropneumonia-like organisms contamination by fluorescence and [^{3}H]thymidine techniques (2, 27).

PE. Known numbers of single cells were plated into replicate 60-mm Petri dishes containing growth medium and were incubated for 2 weeks for colony formation. A cell was considered to have reproductive potential if it gave rise to a colony of 50 or more cells. The ratio of colonies formed to single cells plated is the PE.

T_D Determinations. Known numbers of exponentially growing cells were placed into 60-mm tissue culture dishes containing a known quantity of the growth medium. Once each day for 7 days, replicate plates of cells were sampled. The total cell counts were determined and plotted relative to the sample time, and the length of time required for a cell population to increase by a factor of 2 is the T_D. The experiments were always continued through at least 2 doublings in cell number. The number of cells at which the plateau phase of growth occurs is the saturation density. The average population-doubling times and saturation densities of 4 experiments are reported.

Growth Fraction. The growth fraction represents the proportion of cells actually in the cell cycle (31). Replicate plates of cells in exponential growth were exposed continuously for 4 days to [^{3}H]thymidine (0.5 μCi/ml; specific activity, 1.9 Ci/mmol). Samples were taken 10 min after the radioisotope was added to the cultures and at 24-hr intervals. The cells were removed by trypsinization and centrifuged, and slides were made. Autoradiography using standard liquid emulsion techniques was performed. The fraction of labeled cells at each sample time was determined by microscopic examination. With this procedure, the labeling index increases with time until all cycling cells have passed through S phase. At that time, the labeling index reaches a plateau, and this represents the growth fraction.

Chromosome Counts and Ploidy. Cells in exponential growth were treated with Colcemid (10^{-7} M) for 4 hr. All of the cells were harvested, exposed to a 35-min hypotonic treatment (distilled water:culture medium without serum, 1:1), and fixed with methanol:glacial acetic acid (3:1). Slide preparations of the cells were air dried, stained with Giemsa (4% in Sorensen's buffer, pH 6.8), and scored for the number of chromosomes present in each of 100 metaphase cells. The ploidy of each clone was recorded.

Cell Cycle Phase Distributions and DNA Index. FMF analyses were performed on a Coulter TPS-1 (Coulter Electronics, Hialeah, Fla.). Cells were removed from the tissue culture dishes with pepsin (0.5% for 10 min; Accurate Chemical and Scientific Co., Hicksville, N. Y.). The cells were fixed to a final concentration of 70% ethanol and stored at 4°. To prepare each sample for FMF analysis, an aliquot of cells was stained with ethidium bromide:mithramycin (1:1) for 20 min (52) and run on the FMF (excitation wavelength set at 488 nm). With this procedure, we routinely obtain coefficients of variation ranging from 3 to 5%. The DNA histogram data were analyzed for cell cycle phase distribution by a method developed by Guseman and Bryant (24). A DNA index was determined for each clone from the ratio of peak G_1 channel number for tumor cells to that of the peak G_1 channel number of normal human lymphocytes processed in the same manner using the method of Barlogie *et al.* (1). At least 50,000 cells per clone were quantified for DNA content.

Tumor Growth in Athymic Mice. The ability of the AGS parent line to grow *in vivo* was tested in male BALB/c athymic mice (Harlan Industries, Indianapolis, Ind.). Initially, tissue culture cells were injected s.c. into the right and left flanks of 2 mice (5×10^6 cells/site). Tumor masses were observable in one mouse after 6 weeks, and samples were taken (*a*) for histological examination and comparison with the gastrectomy specimen obtained originally from the patient and (*b*) for transfer to other mice. The tumor is now propagated in the athymic mice, injected as single cells obtained after a 20-min treatment with 1% collagenase at 37°. These cells have a 90% viability as assayed by trypan blue.

RESULTS

Culture Properties. Two to 3 weeks after the biopsy was placed into primary tissue culture, there were obvious areas in the flasks where colonies of cells were growing. Throughout the cultures, the cell morphologies were mixed; round, cuboidal, spindle, and fibroblast shapes were observed, along with an occasional multinucleated cell (Fig. 1*A*). By the fourth week, many individual colonies were visible without the aid of a microscope. With phase-contrast microscopy, some of these colonies were seen to be composed mainly of cuboidal and round cells, and the round cells were usually present as doublets or in groups of 4 cells suggestive of mitotic cells. Several of these colonies were selected, pooled as secondary cultures, and designated as the AGS parent line. After these cells had been in secondary culture for an additional 90 days, they were cloned a second time (Fig. 1*B*). No fibroblast-like cells were present in these cultures, although binucleated cells were seen.

Twelve clones were selected during the second round of cloning and named AGS-1 through AGS-12. Two clones (AGS-5 and AGS-9) failed to thrive. Photomicrographs of stained cells growing on coverslips (5 months in culture) showed them to be oblong to cuboidal (Fig. 1, *C* and *D*). When seeded thinly, the cells tend to spread out and become more flattened (Fig. 1*C*), while in confluent cultures of the same clone (Fig. 1*D*) the cells

take up less surface area. The nuclei are large and lobular, containing many nucleoli.

In the young cultures (30 to 40 days after the biopsy was placed in culture), some colonies grew as shown in Fig. 1*E*. As the cells divided, they arranged themselves in a swirl pattern around a lumen-like clearing in the center, characteristic of cells derived from glandular structures. As the time in culture increased (after 90 to 120 days), these cells were either lost from the cell populations, or the cells themselves were altered and lost this property. The colonies now grow as tight groups of cells (Fig. 1*F*), and as the colonies become larger the cells pile up in multiple layers. The cells have now been in culture for 3 years and are considered as permanent cell lines.

Cell Kinetics and *in Vitro* Growth Properties. The growth characteristics of the AGS parent and 10 clones are shown in Table 1. The T_D of the AGS parent was 24 hr, while the T_D values of the clones ranged from 23 (AGS-3) to 37 hr (AGS-2). The rest of the clones had T_D values grouped between 24 and 30 hr. The cells, initially plated at 2×10^5 cells per 60-mm Petri dish, were grown in Ham's F-10 medium. During the T_D determinations, the cells were never fed again after the start of an experiment. As with other cells tested in this manner (3–5), there was a lag phase (of about 24 hr) before the cell number started to increase. After about 72 hr, the cells depleted the medium of nutrients essential for growth and entered a plateau or stationary phase of growth (7). The cell number reached at this time is called the saturation density. The AGS parent line had a saturation density of 1.6×10^6 cells, with most of the clones also grouped around this value (Table 1). However, 2 clones had much higher values, 2.9×10^6 cells for AGS-4 and 4.5×10^6 cells for AGS-6. No apparent correlation exists between the doubling time of a clone and its saturation density.

The fractions of cells able to traverse the cell cycle (growth fraction) were high, almost 100% in all cases (Table 1). However, fractions of cells able to grow into colonies (PE) were much lower (range, 16 to 70%; Table 1).

The cell cycle phase distributions were determined by FMF analyses on the exponentially growing AGS parent and the clones (Table 2). The variability among the cell cycle phase values for the clones was large, ranging from 40 to 60% cells in G_1, from 29 to 47% for S-phase cells, and from 8 to 21% for G_2M cells.

The DNA index (shown in Table 2) is a measure of the relative DNA content of a cell population compared to a normal standard and was obtained by the method of Barlogie *et al.* (1). A DNA index of 1.0 is characteristic of a diploid population. A DNA index greater than 1.0 generally indicates hyperdiploidy, with 1.5 and 2.0 describing triploid and tetraploid populations, respectively. The DNA index for the AGS parent line was 1.31, and that of the clones ranged from 1.0 to 1.36. This suggests an increase in chromosome number in the cell populations as well but is not necessarily correlated directly with the modal chromosome counts.

Chromosome Complement. The chromosome numbers and patterns are shown in Table 3. One hundred metaphases were analyzed from the AGS parent and each of 7 of the clonal lines. The modal chromosomal numbers were: 45 for clone AGS-1; 46 for clones AGS-8, AGS-11, and AGS-12; 47 for the AGS parent, AGS-6 and AGS-10; and 48 for AGS-4. In a small fraction of cells from each clone, the chromosome numbers ranged from as low as 24 to as high as 92 chromosomes per cell. However, in 75% of the cells studied in each cell line, the chromosome counts were tightly clustered around the modal numbers and ranged only from 43 to 50 chromosomes per cell. There was no apparent correlation between modal chromosome numbers and any of the other culture and cell kinetics properties studied.

Tumor Growth in Athymic Mice. The ability of the AGS parent line to grow *in vivo* was tested in male BALB/c athymic mice. In one of 2 mice initially given s.c. injections in the right and left flanks of AGS parent tissue culture cells, tumors grew to 16 x 26 mm in 6 weeks. One tumor was excised and used to propagate the AGS cells in other athymic mice; the other tumor was excised, examined histologically, and compared to the gastrectomy specimen obtained originally from the parent.

The adenocarcinoma in the gastrectomy specimen varied from focally moderately differentiated with recognizable gland formation to poorly differentiated with sheets, clusters, and cords of malignant cells as the predominant pattern (Fig. 2). The tumor border was of the infiltrative type, and transmural extention into the pancreas was present. Mucin production was slight. Scattered cells contained dense PAS-stained cytoplasmic droplets,

Table 1

Growth characteristics of human adenocarcinoma of the stomach in vitro

Clone	T_D (hr)	Saturation density ($\times 10^6$)	Growth fraction (%)	PE (%)
AGS (parent)	24	1.6	97	70
AGS-1	27	1.7	96	61
AGS-2	37	1.5	98	19
AGS-3	23	1.8	98	16
AGS-4	28	2.9	98	25
AGS-6	27	4.5	99	54
AGS-7	24	2.0	99	41
AGS-8	29	1.5	99	45
AGS-10	27	1.6	99	57
AGS-11	30	1.5	99	36
AGS-12	28	1.8	99	42

Table 2

Cell cycle phase distributions in human adenocarcinoma of the stomach in vitro

Clone	G_1 (%)	S (%)	G_2-M (%)	DNA index
AGS (parent)	60	29	11	1.31
AGS-1	50	40	10	1.26
AGS-2	59	32	9	1.25
AGS-3	45	43	12	1.25
AGS-4	49	30	21	1.36
AGS-6	40	47	13	1.32
AGS-7	52	34	14	1.32
AGS-8	41	45	14	1.25
AGS-10	60	32	8	1.26
AGS-11	42	46	12	1.25
AGS-12	45	46	9	1.0

Table 3

Chromosomal numbers in human adenocarcinoma of the stomach in vitro

Clone	Modal chromosomal no.	Clusters[a]	Overall range
AGS (parent)	47	43–48	39–92
AGS-1	45	42–47	39–87
AGS-4	48	45–49	24–52
AGS-6	47	45–50	38–92
AGS-8	46	43–48	40–52
AGS-10	47	44–48	39–92
AGS-11	46	43–48	39–92
AGS-12	46	43–47	38–48

[a] In 75% of the cells, the chromosome numbers were within this range.

and rare cells had clear vacuoles with faint PAS staining rims (Fig. 2, *arrowhead*). Occasional tumor cells had cytoplasmic mucin droplets which stained with Alcian blue at pH 2.5 (Fig. 2, *curved arrows*).

Tumor tissue grown s.c. in hind legs of athymic mice after transfer from tissue culture was composed of solid nests of oval cells with areas of necrosis. Some cells had PAS-stained dense cytoplasmic mucin droplets (Fig. 3, *double-headed arrow*), and others had clear vacuoles with faintly staining rims (Fig. 3, *arrowhead*). No gland formation or Alcian blue-stained mucin was seen.

DISCUSSION

We have established a parent line and 10 clones from a surgically resected human adenocarcinoma of the stomach as an *in vitro* tumor model system. The *in vitro* growth properties and karyology of the cells have been characterized. In addition, the parent cancer line was shown to grow *in vivo* in athymic mice, and the presence of histochemically identifiable PAS staining cytoplasmic mucin in these cells is evidence that they are epithelial and have the same cytological characteristics as the original malignant cells obtained from the patient.

The present data indicate that the *in vitro* doubling times of the parent and clones range from 23 to 37 hr (Table 1). While the parent and most clones grew until they reached a saturation density of about 1.6×10^6 cells, 2 clones had values which were much higher, 2.9×10^6 (AGS-4) and 4.5×10^6 (AGS-6). Although we report these data only as characteristics of these human stomach cancer cells in culture, there may be relevant reasons for the higher *versus* lower saturation densities, such as differences in nutritional requirements, response to growth hormones, and variations in enzyme levels or metastatic potentials (14, 21, 22, 29, 34, 45, 47), and this will be examined in the future experiments.

The growth fractions were high, almost 100% in all cases. However, colony growth or PE was lower, ranging from 16 to 70% (Table 1). Obviously, almost all of the cells were able to traverse S phase and incorporate [^{3}H]thymidine during a 4-day period required for the GF determination, but when tested for PE (14 days) fewer cells were able to grow into colonies. There are many possible reasons for the lower PEs. (*a*) It might take longer for the cells in some clones to "condition" the medium, and this could lead to unbalanced growth, death, and a reduced PE. (*b*) The cells may be able to divide the 2 to 3 times (in 4 days) required for the growth fraction test, but not be able to do so continuously (about 13 divisions in 14 days) as required for the PE tests. (*c*) Some cells in each clone could in fact differentiate to a point that, although viable, they no longer divide (nonclonogenic) or they divide only with very long cell cycle times. If this were the case, it might be explained by the variability observed among the G_1 values obtained by FMF analyses (Table 2). However, although a large G_1 fraction might suggest the presence of end point or differentiated cells (with a G_1 DNA content), a correlation with lower PEs does not exist. For example, the AGS parent line had a high G_1 fraction (60%; Table 2) but also had a high PE of 70% (Table 1). However, AGS-2 with a much lower PE (only 19%) also had a high G_1 fraction of 59%. It should be remembered that these clones were all isolated from a single tumor biopsy sample and that some or all of these mechanisms may be at work under the selective *in vitro* conditions of a tissue culture system.

The DNA index (Table 2) is a measure of the relative DNA content of a cell population compared to a normal cell standard and was obtained by the method of Barlogie *et al.* (1). A DNA index of 1.0 is characteristic of a diploid population, with 1.5 and 2.0 describing triploid and tetraploid populations, respectively. The DNA index for the AGS parent line was 1.31, and that of the clones ranged from 1.0 to 1.36. A DNA index greater than 1.0 also suggests an increase in chromosome number in cell populations as well but is not necessarily correlated directly with the modal chromosome counts. The chromosome numbers and patterns shown in Table 3 indicated that in a small fraction of cells from each clone the chromosome numbers ranged from as low as 24 to as high as 92 chromosomes per cell. However, in 75% of the cells studied in each clone, the chromosome counts were tightly clustered around the modal numbers and ranged only from 43 to 50 chromosomes per cell. This suggests that the chromosome number has stabilized near the modal values indicated for most of the cells in each clone, but that genetic remodeling continues. This phenomenon could also account in part for the variability in PEs. It is possible that by recloning we may select more stable lines of cells, but it is also likely that this will remain a property of these stomach tumor cells in culture. We have observed similar changes in other long term tissue cultures of Chinese hamster ovary cells (9) and guard against these alterations by replacing the cultures with cells from freezer stocks every 8 weeks. No attempt was made to karyotype or to do banding studies on the cells at this time. There was no apparent correlation between modal chromosome numbers and any of the other culture and cell kinetics properties studied.

It is not unusual to observe differences in these *in vitro* clonal properties since similar data have been reported for other tumor clones *in vitro* (1, 3–5, 21, 22, 41, 44, 45). It is important, however, that such cancer clones be characterized as completely as possible when they are to be used as tumor model systems *in vitro* (3–5, 17–21, 28, 30, 50). To our knowledge, only one other human stomach cancer line exists (Kato III; American Type Tissue Culture Collection, Rockville, Md.), and its characterization is not yet complete. Because the majority of patients who present with carcinoma of the stomach die in a relatively short period of time after the diagnosis is made, it is important that the stomach cancer model system described here be tested further. At this time, studies are underway to determine the *in vivo* growth properties of the other AGS clonal lines in athymic mice and to measure the production of carcinoembryonic antigen. The responses of the parent line and several clones to 8 anticancer agents are completed and will be the subject of another report.[4] When properly characterized, *in vitro* permanent human tumor cell lines serve as excellent model systems on which a variety of differential drug and radiation sensitivities can be studied. The data from such *in vitro* model systems are reproducibly accurate within the model itself and form a basis for comparison with other *in vitro* tumor models. Ultimately, these model systems provide an excellent means of testing the multiple drug and radiation treatment schedules eventually used in human

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tumor therapy (6, 10, 13, 19, 25, 35, 48).

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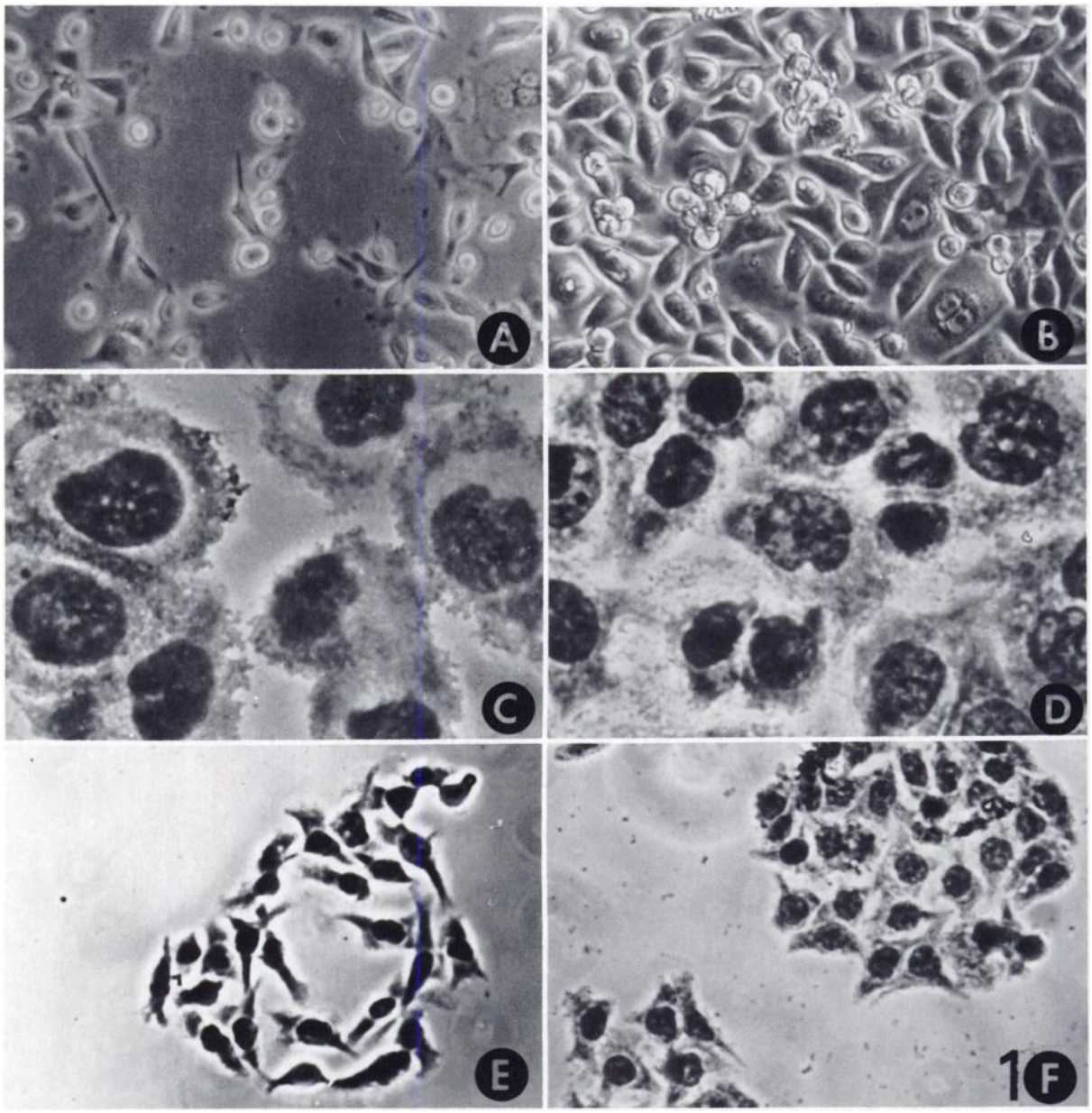
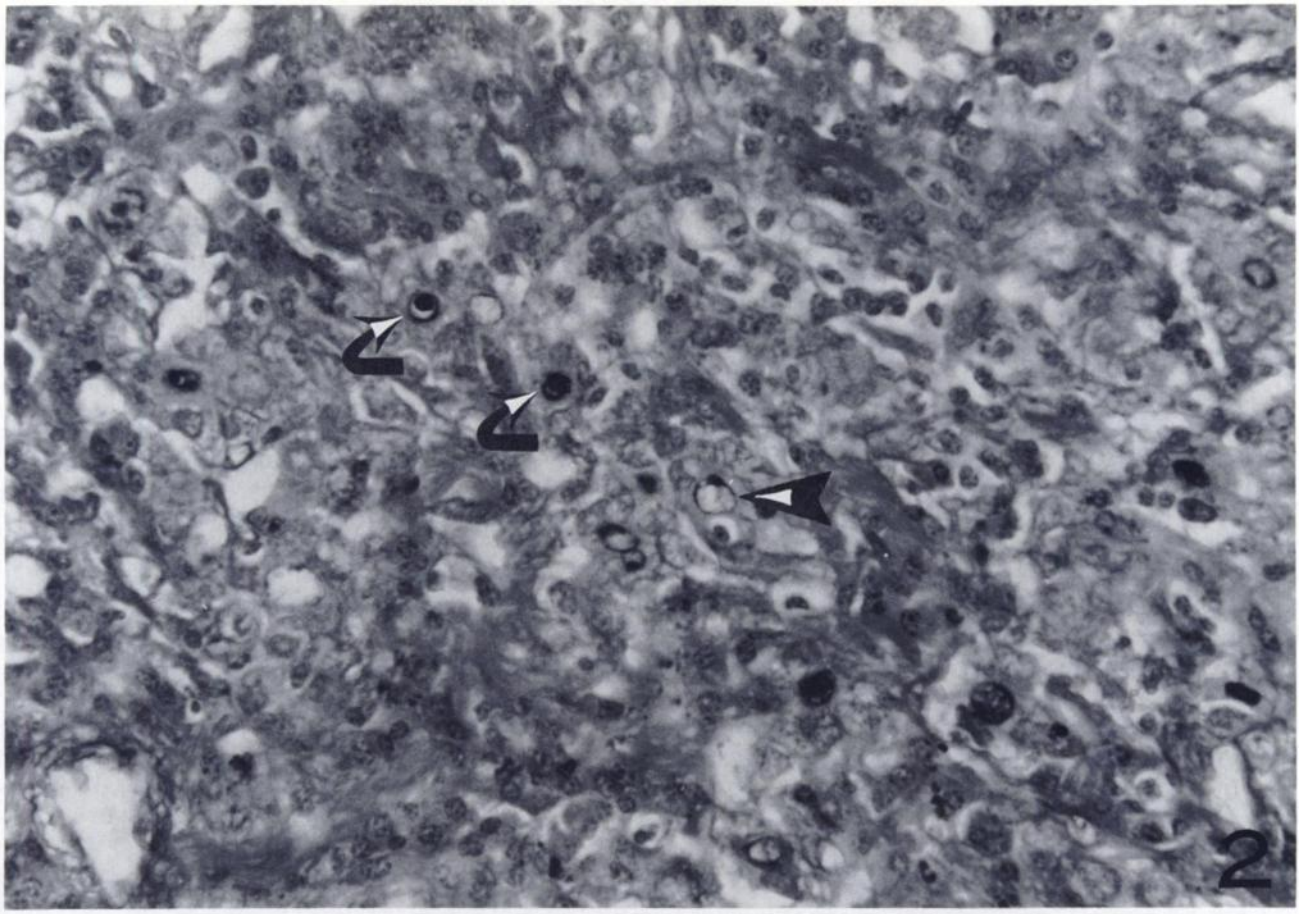


Fig. 1. Photomicrographs of human adenocarcinoma of the stomach cells growing *in vitro*. *A*, phase-contrast microscopy of cells after 3 weeks in primary culture. × 225. *B*, live cells photographed after 4 months in culture. × 225. *C* and *D*, stained cells after 5 months in culture. × 2250. *E*, stained colony of cells in culture for 40 days. × 225. *F*, stained colony after 4 months in culture. × 225.



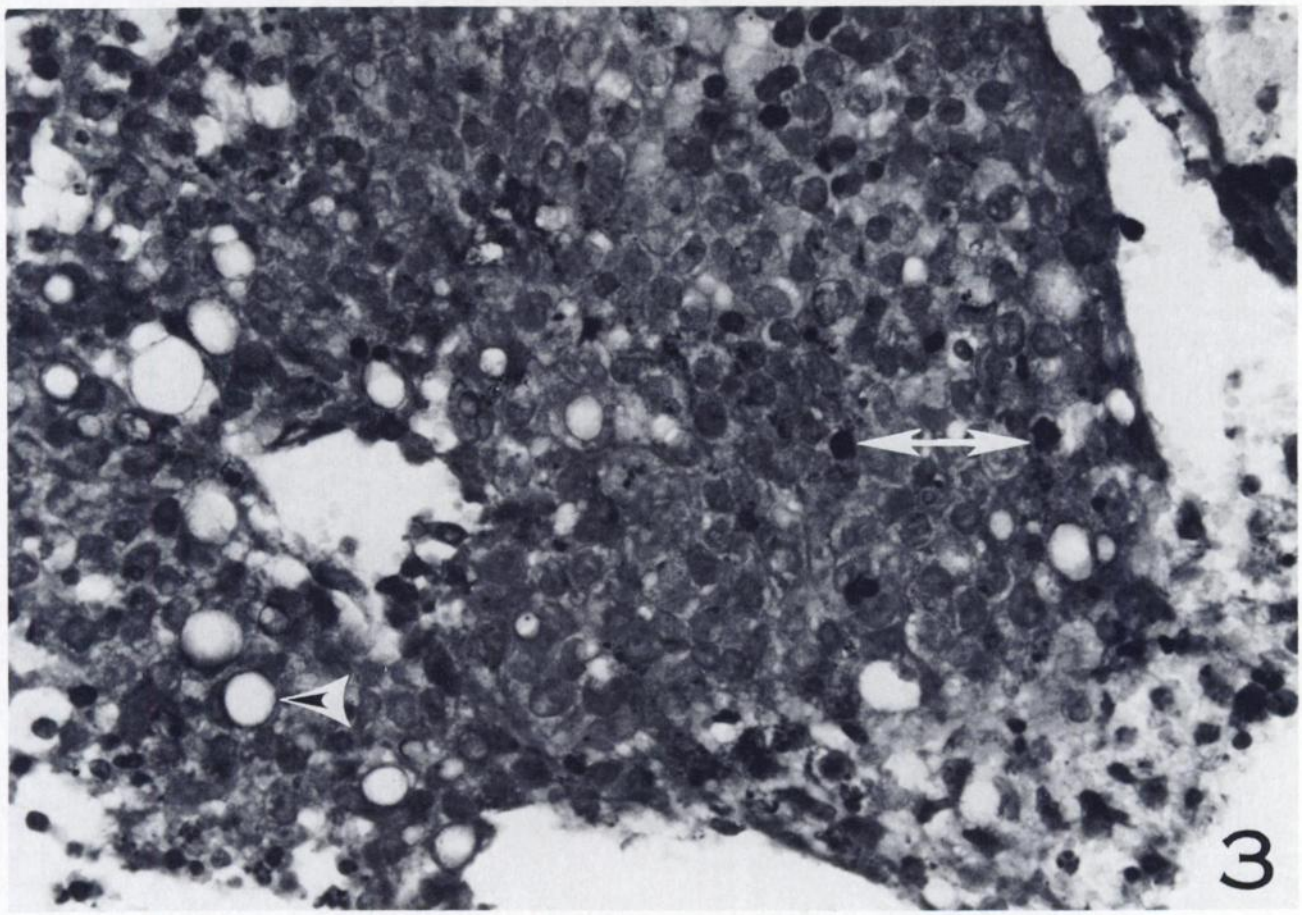


Fig. 2. Photomicrograph of the submucosal area of the gastrectomy specimen infiltrated by nests and cords of poorly differentiated carcinoma cells and many plasma cells. *Curved arrows*, cytoplasmic mucin droplets in tumor cells; *arrowhead*, a carcinoma cell with a vacuole rimmed by PAS-stained material; PAS:Alcian blue (pH 2.5), × 500.

Fig. 3. Photomicrograph of transplanted cells grown in a nude mouse after transfer from tissue culture. A nodule of anaplastic cells has peripheral necrosis (*lower right*); *double-headed arrow*, dense PAS-stained cytoplasmic mucin droplets; *arrowhead*, signet ring-like cell with a clear vacuole partially rimmed by PAS-staining substance indenting the nucleus (PAS:Alcian blue (pH 2.5), × 500.

Establishment and Characterization of an *in Vitro* Model System for Human Adenocarcinoma of the Stomach

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