Heterogeneous Responses of Human Colon Carcinomas to Hexamethylene Bisacetamide

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ABSTRACT

Primary cultures of resected human colon carcinomas were used to study differentiation agents directly on the biologically relevant cancer cells rather than on highly selected established cell lines. To achieve primary cultures which remained viable and replicating for several days, carcinomas were partly digested to epithelial organoids, which were selectively plated with high efficiency on collagen I-bovine serum albumin films in specially formulated serum-free medium. A monoclonal antibody, 29-15, was identified which binds to a cell surface epitope expressed on 16 of 21 invasive colon carcinomas of the Dukes' B2, C, or D histopathology classes, but not expressed on any of 11 noninvasive benign tumors (adenomas) at identical antibody titer. Nontoxic concentrations of the differentiation agent, hexamethylene bisacetamide (HMBA), induced the loss of the 29-15 epitope from HT29 colon carcinoma cells. HMBA also induced HT29 cells to lose the capacity for anchorage-independent growth with a similar dose-response curve and time course to the loss of 29-15 epitope.

Twelve primary cultured human colon carcinomas exhibited differential responses when exposed to 1 to 7 mM HMBA for 7 days. Four moderately to well-differentiated carcinomas lost expression of the 29-15 epitope at each HMBA concentration. The tumor growth fraction was decreased in each tumor, with a mean decrease of 76% at 5 mM HMBA. A dose-dependent induction of nonproliferating tumor colonies, lacking 

INTRODUCTION

The limited success of traditional cytotoxic chemotherapy in the treatment of advanced colorectal cancer has stimulated interest in differentiation therapy (1). The goal of such therapy is to induce colon carcinoma cells to mature to endstage cells which have lost their proliferative and invasive properties. Several investigators have studied the effects of differentiation agents on colon carcinoma cell lines (2-9). These studies clearly demonstrated that the differentiation agents, dimethylformamide, dimethylsulfoxide, and sodium butyrate, could induce a number of morphological, antigenic, and functional changes in several human colon carcinoma cell lines. Dimethylformamide, in particular, induced a loss of anchorage-independent cell growth in vitro and decreased in vivo tumorigenicity in the nude mouse model (5). Established cell lines lack the heterogeneity present within tumors in vivo. This drawback led us to develop a primary culture system with a high efficiency of colony formation from resected colon carcinomas. A large fraction, although not 100% of the carcinoma cells, can be analyzed by this method (see "Materials and Methods"). Rather than utilizing morphological and functional differentiation markers, a MAb was identified which distinguishes malignant cells from their precursors, pre-malignant adenoma cells. Thus we could ask whether a differentiation agent could induce a more benign antigenic phenotype in malignant cells taken directly from the body, and second, whether growth arrest could be induced. By asking these questions, we did not have to judge the extent of morphological or functional differentiation of the treated cells. Permanently differentiated clones of HT29 colon carcinoma cells have been isolated which exhibit many morphological and functional properties of normal, differentiated colonic cells, including cell polarity, active transepithelial transport systems, and mucous secretion (2, 8, 9). These tumor cell lines, although highly differentiated, are not terminally differentiated. They did not exhibit growth arrest and have remained continuous cell lines.

The polar-planar compound HMBA was selected for this study because of its excellent ability to induce differentiation in other systems. HMBA has been shown to be the most potent differentiation agent known of the polar-planar class in Friend murine erythroleukemia cells (10) and human HL-60 promyelocytic leukemia cells (11). HMBA also induced differentiated phenotypes in nonleukemic lines including a human glioblastoma (1, 12) and was shown to modulate ion transport (13). In vivo studies have shown that rats treated with HMBA after receiving the carcinogen methyl-nitrosourea developed fewer mammary tumors with a delay in onset compared with untreated animals (14). The clinical efficacy of HMBA is beginning to be explored with the first Phase I clinical trials recently completed (15, 16). In this study, we asked whether nontoxic concentrations of HMBA could selectively induce loss of a malignancy epitope from human colon carcinoma cells in primary culture, and induce growth arrest.

MATERIALS AND METHODS

Primary Culture of Colon Tumors. Portions of resected carcinomas and adenomas were transported from surgical pathology to the lab in cold DME supplemented with 20 µg/ml gentamicin. Tissues were washed with 0.5% sodium hypochlorite, then three times with wash medium which is DME supplemented with 250 µg/ml streptomycin, 250 µ/ml penicillin, 100 µg/ml gentamicin, 100 µg/ml amakacin, 150 µg/ml chloramphenicol, 1.4 mg/ml bovine serum albumin, 5 µg/ml amphotericin B, and 10 µg/ml tracyclicine (17). The carcinoma cells were dissected free of stroma and any necrotic tissue, and then minced finely to less than 0.5 mm² with two scalpels. The minceate was then washed by rapid agitation to remove the mucous, which prevents organoid attachment, with 5 ml 2% wt/vol acetylclysteine prepared in

1 Supported by NCI grant RO1-CA45783 to E. P., and NCI fellowship CA08161 and a Winston Fellowship to P. C. S.
2 To whom requests for reprints should be addressed, at Sloan-Kettering Cancer Center, Laboratory of Gastrointestinal Cancer Research, P. O. Box 5614, 1275 York Avenue, New York, NY 10021.
3 The abbreviations used are: MAb, monoclonal antibody; HMBA, hexamethylene bisacetamide; DME, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; dThd, thymidine.
Cells were removed by centrifugation at 100 × g for 5 min. Additional washings with acetylcysteine were performed if necessary. If the carcinoma had readily fallen apart into groups of epithelial cells by visual examination by phase microscopy, it was then pushed with a glass pestle through a BSO-μm opening wire mesh placed on a 1910-nm O2, 5% CO2, 92% N2. The growth medium was NCTC 168 (pH 6.7) supplemented with 10 mM selenous acid, 0.29 mM linoleic acid, 0.01 mM deoxycholic acid, 0.1 mM CaCl2, 5 μg/ml pentagastrin, 1 μg/ml hydrocortisone, 5 μg/ml EGF, 20 μg/ml gentamicin, 0.1 U/ml hydrocortisone, 5 μg/ml EGF, 20 μg/ml gentamicin, 0.1 U/ml insulin, 1.7 μg/ml transferrin, 0.1 μM selenous acid, 0.29 μM linoleic acid, 0.01 μM deoxycholic acid, 0.1 mM phosphoethanolamine, and 0.1 mM ethanolamine, and was designated NCTC 168M (18).

Many of the lymphocytes from the lamina propria and other single cells were removed by centrifugation at 100 × g for 2 min. 0.15-ml aliquots of the organoid suspension were placed on 35-mm plates coated for 1–6 h with a mixture of 30 μg/ml of collagen I and 10 μg/ml BSA in NCTC 168. This small volume of medium ensures that each organoid is placed directly on the substrate. The organoids were allowed to attach for 45–60 min in a humidified 5% CO2/air incubator at 37°C. Then 2 ml of medium was carefully added to the side of the dish, and changed 24 h later. The cells were grown in hypoxic conditions at 37°C in 3% O2, 5% CO2, 92% N2. The growth medium was NCTC 168 (pH 6.7) containing no fetal calf serum but 1 mg/ml bovine serum albumin, 0.1 mM CaCl2, 5 μg/ml pentagastrin, 1 μg/ml hydrocortisone, 5 μg/ml EGF, 20 μg/ml gentamicin, 0.1 U/ml hydrocortisone, 5 μg/ml EGF, 20 μg/ml gentamicin, 0.1 U/ml insulin, 1.7 μg/ml transferrin, 0.1 μM selenous acid, 0.29 μM linoleic acid, 0.01 μM deoxycholic acid, 0.1 mM phosphoethanolamine, and 0.1 mM ethanolamine, and was designated NCTC 168M (18).

These methods for the short-term primary culture of epithelial monolayers from normal adult human colonic tissue and from benign and malignant colonic tumors were based on our earlier studies (17–19). The advances in methodology used in the studies in “Results” included the development of a collagen-BSA adhesive matrix, serum-free, highly supplemented medium based on NCTC 168, and hypoxic growth conditions. A tumor “organoid” preparation technique was developed which allowed the epithelial cells of the tumor to remain attached together as small clusters. These organoids rapidly attach to the collagen substrate. Within 24 h the tumor cells migrate in a sheet from the organoids to form monolayer epithelial patches. Depending on the tumor size and organoid yield, the tumor organoids were plated into 40 to 60 35-mm dishes. Organoids isolated from a series of carcinomas were covered with undiluted NTB2 emulsion, exposed in light-tight boxes for 10 days of 4°C, developed, then counterstained with filtered hematoxylin. The radio-labeled and unlabeled nuclei in 12 randomly selected colonies from each of the tumors were counted. A mean of 2900 cells were scored per tumor. This measurement also gave the mean colony size.

Immunological Methods for Primary Cultures. To assay cell-surface epitopes, mouse monoclonal antibodies 29-15 and 29-26 were added at appropriate titers to viable fixed cells in primary culture on plastic Petri dishes as described (19) and the positive cells identified by indirect immunoperoxidase reaction. The negative control was an identical isotype, IgG1, mouse monoclonal MOPC-21. Two investigators rated the intensity of the immunoperoxidase staining relative to controls on the tumor colonies as + to +++ without knowledge of the antibody applied, and the results were averaged.

Monoclonal Antibodies. Mouse M Abs 29-15 and 29-26 were provided in mouse sera with titers of approximately 104. 29-15 is an IgG1 MAb which recognizes a cell surface glycoprotein with a molecular weight of 150,000 unrelated to the A,B,O or Lewis blood group antigens. The serological specificities of this antibody were determined by red blood cell rosetting on a series of established human cancer cell lines and normal cell strains. MAb 29-15 bound to the following carcinoma cell lines: 12 of 17 colon, two of three pancreatic, five of nine breast, four of five lung, one of eight bladder, and two of four ovarian. MAb 29-15 bound weakly to 1 of 15 melanoma cell lines. The MAb did not bind to astrocytoma cell lines (0/13), neuroblastoma cell lines (0/5), renal cell lines (0/2), normal fibroblast strains (0/8), or normal kidney epithelial cell strains (0/2). In frozen section MAB 29-15 bound to the colon, lung, breast, but did not bind to the adjacent normal tissue in 11 cases. It did not bind to a series of normal tissues in section.

Tumor Growth Fraction Measurement by Autoradiography. Standard assay conditions for determining the tumor growth fraction of primary-cultured carcinomas were determined by continuously labeling parallel primary cultures from four carcinomas with 5 μCi/ml, 20 Ci/mmol [3H]dThd. Multiple points for analysis were taken between 1 and 72 h. A plateau value was reached between 24 and 72 h of labeling (Fig. 1). Detachment of labeled cells only occurred when the labeling continued for 5 or more days (data not shown). A plateau value of [3H]dThd-labeled cells was reached because the high level of radioactivity arrested the colony growth of primary cultures but did not arrest the adjacent normal tissue in 11 cases. It did not bind to a series of normal tissues in section.

Pathology Diagnosis. The histology of each carcinoma was reviewed by Dr. Carlos Urmacher of the Department of Pathology, Memorial Hospital, Memorial Sloan-Kettering Cancer Center. The carcinomas tested in primary culture with HMBA were selected from eight men and four women, ages 45 to 75, mean age 65.6 years. The tumor sites were one rectal, seven sigmoid, one ascending colon, two caecal, and one hepatic metastasis.

Fig. 1. Tumor growth fraction of colon tumors placed into primary culture. Parallel primary cultures were plated from tumors labeled "A", "B", "C" etc. and the tumor growth fraction determined at each time point (see "Materials and Methods"). Mean and standard error bars are shown.
Cell Line Culture. HT29 colon carcinoma cells were originally received from Dr. Jorgen Fogh who had initiated the line. The cells were cultured in DME at pH 7.4 supplemented with 7% newborn bovine serum, 20 μg/ml gentamicin, 4 mM glutamine, and buffered with 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, with the NaHCO₃ concentration of the DME decreased to 1.2 g/liter to prevent hyperosmolality. The final osmolality was 287 ± 10 mosmol. For better attachment of the cells to glass coverslips used in the immunofluorescence studies, the coverslips were coated with 0.1% gelatin.

Soft Agar Cloning. This was performed in DME using 3 ml of a 1% seaplaque agarose underlayer and a 2-ml 0.6% agarose overlay prepared in 60-mm Corning Petri dishes with a 2-mm grid for ease of scoring colonies. All colonies over 0.1 mm (2.5-μm actual diameter) were counted after individual measurement using a grided micrometer placed in the eyepiece of an inverted phase microscope. Colonies were scored after 13 days of growth in a 5% CO₂/air incubator. When seeded at 1000 cells per plate, the Kirsten-virus transformed 3T3 cell line, the negative control, gave no colonies.

Immunofluorescence Techniques. Binding of MAbs 29-15, 29-26 and the isotype control for these IgG; MAb, MOPC21, to cell surfaces was measured by adding the MAbs to cells grown on glass discs after three washes with phosphate-buffered saline containing 0.1% bovine serum albumin and no permeabilization step. MOPC21 was added at 20 μg/ml (19). After binding for 45 min at room temperature in a humidified chamber, the cells were washed as above and a secondary antibody, fluorescein-conjugated IgG fraction goat antimouse immunoglobulins (IgA&IgG&IgM, H & L chains, Cappel Labs), was applied for an identical incubation at a 1/20 dilution after reconstitution according to the supplier’s instructions. Fluorescence photomicrographs of 10 random fields were taken using first a 40X epifluorescence objective, then a 40X phase objective, so the total cells per field could be counted. All cells with a punctate border of fluorescence were graded as positive.

RESULTS
Identification of Malignancy Marker. The use of unpermeabilized viable cells in primary culture 24 h after plating allowed the identification of MAbs which bound only to the cell surface of tumor cells. Twenty-one invasive carcinomas (Dukes’ B2, C, and D classes) received from surgical resections and 11 adenomatous polyps (premalignant noninvasive colon tumors) were screened for their level of cell surface expression of the 29-15 epitope. Twenty-one invasive carcinomas continued to express the 29-15 epitope by indirect immunoperoxidase assay (see “Materials and Methods”) using MAb 29-15 dilutions of 10⁻², 10⁻³, and 10⁻⁴ M Ab dilution (Table 1). The epitope was more prevalent on the carcinoma cells than on the adenoma cells. When MAb 29-15 was diluted to 10⁻⁴, 76% (16 of 21) of the carcinomas continued to express the epitope while none of the 11 adenomas did. Thus the noninvasive benign tumors, the adenomas, could be distinguished from the majority of invasive carcinomas by their lower level of expression of the 29-15 cell surface glycoprotein.

Decrease in 29-15 Epitope Expression of Cell Line HT29 but Lack of Induction of Growth Arrest by HMBA. When the human colon carcinoma cell line HT29 was treated with the differentiation inducer HMBA at 1.0 to 2.0 mM for 4–8 days, the cells exhibited a dose-dependent decrease in growth rate (Fig. 2). The trypan blue exclusion assay, when performed on HT29 cells treated with 1 to 10 mM HMBA, demonstrated that <5% of cells treated with 1–5 mM HMBA had membranes so damaged that they were unable to exclude the dye. These data, given the limitations of the trypan blue exclusion assay, suggest that the growth inhibition was not due to cytotoxic effects of HMBA. The HMBA-treated cells were not growth-arrested but continued to proliferate more slowly than untreated cells. All HMBA-treated cells incorporated [³H]thymidine into their nuclei during 48 h of continuous labeling but with slower kinetics than untreated cells (autoradiography data not shown).

HMBA from 0.1 to 1.5 mM induced a dose-dependent loss of the 29-15 epitope (Fig. 3). After treatment with 1.5 mM HMBA, the fraction of HT29 cells expressing this epitope was decreased about 20-fold (Table 2). Cells expressing the 29-15 epitope displayed a punctate cell surface fluorescence (Fig. 4) which was lost after HMBA treatment (Fig. 4B). In a control experiment, no modulation of 29-15 epitope expression was observed when cells were grown to different densities (data not shown). Thus the effects on 29-15 expression were not due to the lower saturation density achieved in HMBA-containing medium.

Because HMBA is known to alter a number of membrane properties (13, 21), the expression of an unrelated pancolonic cell surface epitope identified by MAb 29-26 (see “Materials and Methods”) was used as a control for loss of 29-15 epitope expression. The fraction of cells which expressed the 29-26 epitope decreased only marginally with HMBA treatment (Ta-
dishes were ready. The secondary antibody was then applied to all dishes. At each dishes onto gelatin-coated glass cover slips. After attachment overnight, 1.5 mM control MAb, MOPC21, was uniformly negative on both control and treated

cells which had at least half of their circumference marked by punctate fluores
citation of the number of tumor colonies that were induced to
8 days (Fig. 3, Table 2).

dependent Growth and Loss of the 29-15 Cell Surface Malig

time point the pancolonic-positive control MAb 29-26, and an isotype identical bound antibody were methanol fixed and stored in methanol until the last set of
test 

Table 2

<table>
<thead>
<tr>
<th>Monoclonal used</th>
<th>Antigen-positive cells (%)</th>
<th>Total cells</th>
<th>Total fields</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancolonic MAb 29-26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>On control cells</td>
<td>97.1 ± 0.5</td>
<td>512</td>
<td>2</td>
</tr>
<tr>
<td>On HMBA-treated cells</td>
<td>88.8 ± 3.0</td>
<td>766</td>
<td>5</td>
</tr>
<tr>
<td>Malignancy marker MAb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29-15 tested</td>
<td>43.9 ± 7.7</td>
<td>2013</td>
<td>10</td>
</tr>
<tr>
<td>On control cells</td>
<td>1.7 ± 0.9</td>
<td>2596</td>
<td>14</td>
</tr>
</tbody>
</table>

2). Therefore, HMBA treatment did not modulate all cell surface epitopes, but specifically induced loss of the 29-15 malignancy marker.

Parallel Kinetics for HMBA-induced Loss of Anchorage-independent Growth and Loss of the 29-15 Cell Surface Malignancy Epitope. HT29 cells treated with HMBA exhibited loss of anchorage-independent growth which became apparent after 2 days. This loss continued until a plateau value was reached after 5–8 days of HMBA treatment (Fig. 5, data not shown). Similarly, loss of 29-15 epitope expression was only seen after 2 days of HMBA treatment and the maximum effect after 4 to 8 days (Fig. 3, Table 2).

Methods of Primary Culture Analysis of Colon Tumors. Four methods used to analyze the effects of HMBA were: measurement of the expression of the 29-15 epitope by serial dilutions as was done in the screening study shown in Table 1; enumeration of the number of tumor colonies that were induced to growth arrest; measurement of the mean colony size; measurement of the tumor growth fraction ("Materials and Methods"). Established colon carcinoma lines studied consisted of greater than 99% of cells in cycle (data not shown). In contrast, the carcinomas from resections were composed of a nonproliferating fraction and a tumor growth fraction, both in vivo and in vitro. The proliferative fractions measured in the tumors in this study ranged from 2 to 54% of the total cells. Mass culture methods such as total DNA content or total cell number were thought not be sensitive enough to show differences in short-term studies of tumors with low growth fractions.

Effects of HMBA on Primary Cultured Colon Carcinomas. Twelve primary cultured colon carcinomas in multiple sets of 35-mm dishes were continuously exposed to 0, 1, 3, 5, or 7 mM HMBA for 7 days, and the tumor growth fraction determined ("Materials and Methods"). One set of parallel primary cultures from each tumor was assayed for 29-15 epitope expression by dilutions of the MAb at each HMBA concentration, with appropriate positive and negative MAb controls ("Materials and Methods"). The studies with the HT29 cell line predicted that HMBA would induce loss of the 29-15 epitope in susceptible tumors, but would not induce growth arrest. However, HMBA induced three different response patterns in the primary-cultured carcinomas: (a) decrease in the tumor growth fraction with induction of growth-arrested colonies and concomitant loss of 29-15 expression, (b) no effect on either tumor growth fraction or 29-15 epitope expression, and a totally unexpected result (c) a 2.5-fold mean increase in the tumor growth fraction, a 90% mean increase in tumor colony size, and no decrease in 29-15 epitope expression. A summary of the varied effects of HMBA on the carcinoma growth fractions is shown in Fig. 6.

Growth-arrested tumor cells lacking cell surface 29-15 epitope expression were induced by HMBA in four moderate to well differentiated carcinomas (Table 3). Tumor colonies consisting of only growth-arrested cells were induced by HMBA with an optimum at 5 mM in three of these carcinomas: 1042, 1136, and 1143 (Fig. 7). Carcinoma 1042 consisted of two colony types: large colonies (262 ± 95 cells) containing proliferating, [3H]dThd labeled cells and smaller colonies (67 ± 13 cells) containing only nonproliferating cells. 40% of untreated carcinoma 1042 primary cultures were small growth-arrested colonies after 7 days of culture. HMBA from 1 to 5 mM induced a linear increase in the proportion of these small, growth-arrested colonies from 58% to 96% (Fig. 7). As a result the tumor growth fraction was decreased over 50-fold to 0.03% by 5 mM HMBA (Table 4). 29-15 epitope expression was completely inhibited at all HMBA concentrations.

HMBA also induced a dose-dependent growth-arrest of cells in tumors 1136 and 1143 (Fig. 7, Table 3), and in tumor 1116 (Fig. 8) with parallel loss of 29-15 expression. Tumors 1136, 1143, and 1116, if left untreated for 7 days, had no growth-arrested colonies. 7 days of 5 mM HMBA treatment induced loss of all proliferating cells in 70% of carcinoma 1136 colonies and in 46% of carcinoma 1143 colonies, causing 50- and 35-fold decreases in their tumor growth fractions, respectively (Table 4). HMBA induced fewer cells to growth-arrest in primary cultured carcinoma 1116 (not to be confused with the cell line SW1116). No HMBA-treated tumor colonies were induced to lose all of their dividing cells. However, HMBA from 3 to 7 mM induced a decrease from 54 to 36% in the fraction of proliferating cells (Table 4), and a parallel loss of 29-15 expression (Fig. 8). In two other carcinomas, 1046 and 1160, HMBA induced no change in any of the parameters examined (Tables 3 and 4, Fig. 6).

In six other primary cultured colon carcinomas HMBA had an unexpected growth stimulatory effect. A two- to threefold growth increase was observed after 7 days (Fig. 8), with parallel loss of 29-15 epitope expression. The proliferative fractions measured in the tumors in this study ranged from 2 to 54% of the total cells. Mass culture methods such as total DNA content or total cell number were thought not be sensitive enough to show differences in short-term studies of tumors with low growth fractions.

HETEROGENSE RESPONSES OF HUMAN COLON CARCINOMAS TO HMBA

Fig. 3. Dose-dependent inhibition of expression of 29-15 epitope by HMBA. HT29 cells were seeded at 3 x 10⁴/100-mm dish, in 30 parallel dishes. 24 h after seeding HMBA was added to one dish and the cells allowed to grow for 8 days. The cells were assayed for cell surface determinants by leaving them untreated, not permeabilized (see Materials and Methods) using the MAb listed. The isotype control MAb, MOPC21, was uniformly negative on both control and treated cells. Random fields were photographed by epifluorescent microscopy and by phase contrast, and printed as 3- x 4-inch photographs. These were scored for cells which had at least half of their circumference marked by punctate fluorescence. There were a mean of 210 and 177 cells on control fields and HMBA-treated fields, respectively.
HETEROGENEOUS RESPONSES OF HUMAN COLON CARCINOMAS TO HMBA

Fig. 4. Inhibition of expression of 29-15 cell surface epitope by HMBA. HT29 carcinoma cells were cultured in the presence of 1.5 mM HMBA for 8 days, then binding of MAb 29-15 was performed on nonpermeabilized cells to detect only cell surface epitopes using indirect immunofluorescence assay (see "Materials and Methods"). The cells were overlaid with a 1:10 dilution of MAb 29-15 serum stock of titer 10^7, the dilution discriminating between adenomas and carcinomas. A, control cells; B, HMBA-treated cells. This shot was taken with a much longer exposure than "A" to allow some visualization of the cells. Final magnification, x256.

Fig. 5. Time course for induction of loss of anchorage-independent growth by HT29 carcinoma cells treated with 1.0 mM HMBA. Parallel T-25 flasks with exponentially growing HT29 cells seeded at 4 x 10^4 cells per flask were either treated with 1.0 mM HMBA or grown in its absence. Then 1000 cells were seeded into agarose (see "Materials and Methods"). The percentage of untreated cells growing in agarose (6.9 ± 0.4% of input) was normalized to 100%. The percentage of HMBA-treated cells that grew to colonies in agarose was plotted as a fraction of control values. Loss of anchorage-dependent growth was seen over a range of 200 to 5000 HMBA-treated cells placed in agarose per plate, with the inhibition more pronounced at the lower cell numbers (data not shown).

increase in the tumor growth fraction was induced (Table 4, Fig. 6), tumor colony size was increased (Fig. 9), and no loss of 29-15 epitope expression was observed (Fig. 10). Cells treated with only 1 mM HMBA exhibited no increase in growth fraction compared with controls and grew with the same kinetics as untreated cells (Fig. 9). Primary-cultured tumor 1132 also exhibited growth stimulation only at 3–7 mM (Fig. 10), with no effect at 1 mM. The mean tumor colony size was increased statistically at 5 mM HMBA (Table 3), also demonstrating growth stimulation. The 29-15 epitope expression was not decreased at any HMBA concentration tested (Fig. 10). The tumor growth fraction was increased statistically at 5 mM in each of the six tumors of this group (Table 4). An increase in mean colony size was observed in each of these six carcinomas treated with 5 mM HMBA, but only in three was it as well as a 279% increase in the tumor growth fraction (Table 4). Cells treated with only 1 mM HMBA exhibited no increase in growth fraction compared with controls and grew with the same kinetics as untreated cells (Fig. 9). Primary-cultured tumor 1132 also exhibited growth stimulation only at 3–7 mM (Fig. 10), with no effect at 1 mM. The mean tumor colony size was increased statistically at 5 mM HMBA (Table 3), also demonstrating growth stimulation. The 29-15 epitope expression was not decreased at any HMBA concentration tested (Fig. 10). The tumor growth fraction was increased statistically at 5 mM in each of the six tumors of this group (Table 4). An increase in mean colony size was observed in each of these six carcinomas treated with 5 mM HMBA, but only in three was it
HETEROGENEOUS RESPONSES OF HUMAN COLON CARCINOMAS TO HMBA

Table 3 Effects of 5 mM HMBA on primary-cultured carcinomas

<table>
<thead>
<tr>
<th>Epitope Expression</th>
<th>Tumor Growth Fraction</th>
<th>Mean Colony Size</th>
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<tbody>
<tr>
<td>Carcinomas induced to growth arrest</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderately differentiated colon carcinoma 1042</td>
<td>Decreased to adenoma level</td>
<td>Decreased to 2% of control with 96% of colonies growth arrested</td>
</tr>
<tr>
<td>Moderately differentiated colon carcinoma 1136</td>
<td>Decreased to adenoma level</td>
<td>Decreased to 2% of control with 70% of colonies growth arrested</td>
</tr>
<tr>
<td>Moderately differentiated colon carcinoma 1116</td>
<td>Decreased to adenoma level</td>
<td>Decreased to 61% of control no colonies growth arrested</td>
</tr>
<tr>
<td>Well-differentiated colon carcinoma 1143</td>
<td>Decreased to adenoma level</td>
<td>Decreased to 30% of control with 46% of colonies growth arrested</td>
</tr>
<tr>
<td>Carcinomas stimulated to divide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderately differentiated colon carcinoma 1132</td>
<td>Unaffected</td>
<td>Increased 210%</td>
</tr>
<tr>
<td>Poorly differentiated colon carcinoma 1048</td>
<td>Unaffected</td>
<td>Increased 329%</td>
</tr>
<tr>
<td>Poorly differentiated colon carcinoma 1055</td>
<td>Unaffected</td>
<td>Increased 277%</td>
</tr>
<tr>
<td>Moderately differentiated colon carcinoma 1153</td>
<td>Unaffected</td>
<td>Increased 306%</td>
</tr>
<tr>
<td>Moderately differentiated colon carcinoma 1161</td>
<td>Unaffected</td>
<td>Increased 279%</td>
</tr>
<tr>
<td>Moderately differentiated colon carcinoma 1233</td>
<td>Unaffected</td>
<td>Increased 190%*</td>
</tr>
<tr>
<td>Carcinomas unaffected by HMBA</td>
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<td></td>
</tr>
<tr>
<td>Moderately differentiated colon carcinoma 1160*</td>
<td>Unaffected</td>
<td>Unaffected</td>
</tr>
<tr>
<td>Colloid colon carcinoma 1046</td>
<td>Unaffected</td>
<td>Unaffected</td>
</tr>
</tbody>
</table>

* Peak value at 3 mM HMBA.
* Only 5 mM tested.

statistically significant, possibly because of the short duration of the experiment (summarized in Table 3).

DIscussion

HMBA-induced growth arrest, as measured by lack of [3H]-dThd incorporation, coupled with loss of a cell surface malignancy marker in four of 12 resected colon carcinomas assayed in primary culture. All of the HMBA growth-arrested carcinomas were well to moderately differentiated in vivo, suggesting that HMBA was able to induce growth arrest only in tumor cells already at advanced stages of differentiation. Under the same primary culture conditions, HMBA induced an unexpected threefold increase in the tumor growth fraction, a doubling of tumor colony size, and no loss of the malignancy cell surface marker in another set of six tumors. These carcinomas looked very healthy in five and seven mM HMBA with colony growth apparent. One carcinoma could be passaged after HMBA treatment, while the parallel untreated cultures died and detached from the plate. This group of growth-stimulated carcinomas had initial growth fractions of 4 to 24% (Table 4), similar to the initial growth fractions of the group induced to growth arrest (1.6–53.8%). The factor which distinguished between the carcinoma groups was that the growth-stimulated carcinomas were poorly to moderately differentiated, as based on pathology reports ("Materials and Methods"), less differ-

Fig. 7. Induction of tumor colonies containing only growth-arrested cells in three colon carcinomas by HMBA. The percentage of nondividing colonies induced by a 7-day continuous exposure to HMBA from 1–7 mM is plotted.
Carcinomas unaffected by this treatment. A mean of 23 tumor colonies was evaluated for each data point. P values <0.05 were considered statistically significantly different.

Table 4 Changes in tumor growth fraction induced by 7-day exposure to 5 mM HMBA

<table>
<thead>
<tr>
<th>Tumor no.</th>
<th>Untreated mean ± SE</th>
<th>HMBA-treated mean ± SE</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinomas induced to growth arrest</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1042</td>
<td>1.6 ± 0.4%</td>
<td>0.03 ± 0.02%</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>1136</td>
<td>20.0 ± 3.5%</td>
<td>0.4 ± 0.2%</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>1116</td>
<td>53.8 ± 4.9%</td>
<td>36.0 ± 3.9%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>1143</td>
<td>24.7 ± 5.4%</td>
<td>0.7 ± 3.4%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Carcinomas induced to divide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1132</td>
<td>22.8 ± 3.7%</td>
<td>47.8 ± 6.0%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>1048</td>
<td>6.6 ± 2.7%</td>
<td>21.7 ± 8.7%</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>1055</td>
<td>12.3 ± 2.4%</td>
<td>35.1 ± 7.2%</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>1116</td>
<td>18.0 ± 2.0%</td>
<td>55.0 ± 6.0%</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>1161</td>
<td>3.8 ± 1.5%</td>
<td>10.6 ± 3.8%</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>1233</td>
<td>23.8 ± 5.3%</td>
<td>45.2 ± 3.7%</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Carcinomas unaffected by this exposure to HMBA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1160</td>
<td>13.2 ± 3.0%</td>
<td>7.1 ± 4.4%</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>1046</td>
<td>9.6 ± 3.8%</td>
<td>7.9 ± 2.4%</td>
<td>&gt;0.1</td>
</tr>
</tbody>
</table>

* Tested at 7 mM HMBA.  
* Experiment terminated after 5-day exposure.

Fig. 8. Correlation between inhibition of tumor cell growth and loss of 29-15 epitope expression. Primary Tumor 1116 was placed into parallel cultures in 35-mm dishes and the effects of HMBA at 1, 3, 5, and 7 mM HMBA for 7 days assayed on 29-15 expression and tumor growth fractions (see "Materials and Methods").

Fig. 9. Increase in mean colony size of Tumor 1161 treated with 5 mM HMBA. Parallel primary cultures of colon carcinoma 1161 were grown in the presence of 1 mM HMBA, 5 mM HMBA, or left untreated. Plates were taken after 1, 3, and 5 days of growth, and the mean colony size ± standard error determined ("Materials and Methods").

entiated on average than the group growth arrested by HMBA. Perhaps HMBA induces both types of carcinoma cells to differentiate. By this hypothesis only the carcinomas already well-differentiated can be induced to growth arrest within the 7-day treatment period, whereas the poorly differentiated carcinomas would need a longer treatment time since they would have to pass through additional differentiation stages. There is precedent for this interpretation. Cells at intermediate stages in erythrocyte differentiation continue to divide as they differentiate (23). Treatment with erythropoietin and colony-stimulating factors induced cell division and continued differentiation of burst-forming units into the next stage, colony-forming units. Colony-forming units then can be further differentiated to the terminal stage, nondividing erythrocytes.

Another rather unexpected result of this study was that the established carcinoma cell line HT29 was not an accurate model for any of the response patterns of the resected tumors. In HT29 cells 29-15 epitope expression was inhibited without a parallel decrease in growth fraction as measured under anchorage-dependent growth conditions (on plastic). HT29 cells did show some restriction in growth potential with 5–8 days of HMBA treatment, losing their ability for growth in agarose. The kinds of aberrant growth controls seen in resected tumors, which are composed of interacting cell populations (24), may be very different from the kinds of aberrant growth controls selected for when individual carcinoma cells become established lines, and thus may present different targets to differentiation agents. An alternative explanation for the lack of correlation of the HT29 cell line results with the resected tumors is that the cell lines were always treated with low concentrations of HMBA, 1.0–1.5 mM for at most 8 days. A 3-week exposure to 5 mM HMBA did induce differentiated phenotypes of fluid-transporting cells and mucin-producing cells in the HT29 cell line (Schroy and Friedman, data not shown), as reported for long term exposure of HT29 cells to sodium butyrate (2, 9). We thus speculate that loss of the 29-15 cell surface epitope expression is a very early marker of one of these differentiation pathways.

In conclusion, HMBA induced a varied pattern of responses in primary-cultured colon carcinomas. Since about 75% of all carcinomas received in the laboratory can be studied in primary culture (19, 20), these in vitro results are representative of the patient population. Further studies will be aimed at elucidating the mechanism of these different responses, and at developing additional markers to identify carcinomas that can be growth arrested by HMBA. Ideally, such markers could be assayed for in fixed tissue sections which are routinely prepared from the primary tumor at the time of surgery. This would allow a
rational selection of patients for differentiation therapy for the treatment of advanced colon cancer.

REFERENCES


Heterogeneous Responses of Human Colon Carcinomas to Hexamethylene Bisacetamide


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