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Effects of Sodium Butyrate and Dimethylsulfoxide on Human Pancreatic Tumor Cell Lines*

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Abstract—*The effects of* 1 mM *sodium butyrate or* 2% *dimethylsulfoxide (DMSO) on three human pancreatic tumor cell lines were examined. The cell lines tested were MIA PaCa-2, PANC-1 and CAPAN-1. Both butyrate and DMSO inhibited the ability of all three lines to form colonies in soft agar. These results suggest that the use of these agents provides a model system for the study of the molecular changes involved in human pancreatic cancer. In butyrate all the cell lines showed a marked increase in cellular levels of alkaline phosphatase, while growth in DMSO led to a reduction in most cases. DMSO caused a rapid reduction in the attachment of all three cell lines to collagen substrates, while butyrate had no effect. These results illustrate the fact that although both butyrate and DMSO appear to greatly reduce the parameters correlated with tumorigenicity of human pancreatic cancer cells, the mechanisms involved may be very different.*

INTRODUCTION

ONE APPROACH in studying the molecular mechanisms of tumor formation is to treat cancer cells in culture with agents which will reduce their tumorigenicity and to measure the concomitant changes which occur in cellular characteristics. In view of the hypothesis that cancer is related to aberrant differentiation [1], it is no surprise that compounds which are capable of inducing differentiation in erythroleukemic cells [2] will reduce the malignant properties of cancer cell lines [3]. In many cases this effect is reversible upon removal of the inducing agent [4]. For this reason measurement of the tumorigenicity of the treated cells *in vivo* is difficult and a simpler approach is to measure the tumorigenicity *in vitro* by the ability of the cells to grow in an anchorage-independent manner. Although the correlation between anchorage-independent growth and tumorigenicity is not absolute [5], if the cell lines are known to be both tumorigenic and capable of anchorage-independent growth, inhibition of the latter property very probably reflects a reduction in tumorigenicity also. In recent studies with human colon cancer cell lines it was found that both butyrate and dimethylsulfoxide (DMSO) reduced the tumorigenicity of the cancer cell lines as measured by growth in soft agar [4]. *N,N*-Dimethylformamide also inhibited growth of human colon cancer cells in soft agar and reduced the tumor formation by the cells in athymic nude mice [3, 6]. In both studies [4, 6] the loss of malignant properties was accompanied by the appearance of markers of a more differentiated phenotype [7].

This approach is potentially very useful in the study of human pancreatic cancer. Although the lack of normal cell lines with which to compare lines established from human tumors has been a problem with many types of cancers, in most cases the properties of normal human tissue could be investigated. With pancreatic cancer, however, a large percentage of the carcinomas are thought to originate in the ductal cells of the organ [8], which account for only 4% of the volume of the pancreas [9]. Moreover, isolation and biochemical analysis of pancreatic ductal tissue is a complex procedure [10, 11]. Because of the difficulty in comparing human pancreatic cancer cell lines with normal cells, the effects of sodium butyrate and DMSO on three such cell lines were examined in order to

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establish a system in which the molecular changes related to malignancy can be investigated.

MATERIALS AND METHODS

Cell lines

MIA PaCa-2 cells were provided by Dr Adel A. Yunis (University of Miami School of Medicine, Miami, FL). The PANC-1 cells were obtained from Dr Walter A. Nelson-Rees and were produced with support from the National Cancer Institute, Viral Oncology Program, under the auspices of the Office of Naval Research and the Regents of the University of California. The CAPAN-1 cells were provided by Dr Jorgen Fogh (Sloan-Kettering Institute for Cancer Research, Rye, NY). The cell lines were all maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml).

Growth curves in the absence and presence of butyrate or DMSO were produced by seeding the cells at a density of 1×10^5 cells per dish in 35-mm plastic Petri dishes in growth medium with or without the agent to be tested and the plates were incubated at 37°C in 95% air–5% CO_2. At daily intervals duplicate plates were carefully washed with 0.01 M sodium phosphate–0.15 M sodium chloride (pH 7.6). The attached cells were removed with 0.05% trypsin–0.02% EDTA and the number of cells was determined using an electronic cell counter (Coulter Electronics, Hialeah, FL). In all experiments involving DMSO or butyrate the final concentrations of the agents in the growth medium were 2% DMSO or 1 mM sodium butyrate.

Growth in soft agar

To determine the efficiency of colony formation in soft agar, cells were harvested and suspended at a density of 5×10^3 or 5×10^4 cells/ml in 0.3% agar (Difco Laboratories, Detroit, MI) in growth medium or in growth medium containing 2% DMSO or 1 mM butyrate. One milliliter of the suspension was then layered on 1 ml of 0.5% agar which had already been allowed to harden in a 35-mm plastic Petri dish. The 0.5% agar was made up in the same medium as the upper layer. Cells which had been grown in 2% DMSO or 1 mM butyrate for 8 days were also harvested and assayed in the same manner.

In all cases the cultures were incubated at 37°C in 95% air–5% CO_2. After 24 hr incubation all the plates were inspected to ensure cells were suspended as single cells. The plates were fed after 7 days with 1 ml of the appropriate medium and the number of colonies was determined after 16 and 28 days in each case using a light microscope.

Enzyme assays

For enzyme assays, cells grown for at least 8 days in the absence or presence of 2% DMSO or 1 mM sodium butyrate were washed twice with 0.01 M sodium phosphate–0.15 M sodium chloride (pH 7.4), scraped from the flask, spun down and resuspended in the same buffer. The cells were sonicated for 20 sec and the sonicate was taken for further assays.

Alkaline phosphatase (EC 3.1.3.1) activity was determined in 1 ml reaction mixture containing 5 mM *p*-nitrophenyl phosphate (Sigma, St. Louis, MO), 0.75 M 2-amino-2-methyl-1-propanol (pH 10.3) and 1 mM $MgCl_2$. The reaction was carried out at 37°C and was stopped by the addition of 1 ml of 1 M NaOH. The product released was determined by measuring the absorbance at 410 nm. Blank values were obtained by adding 1 M NaOH to the reaction mixture at zero time.

Amylase (EC 3.2.1.1) activity was determined using a test kit obtained from Pharmacia Diagnostics, Piscataway, NJ. Protein was determined by the method of Lowry *et al.* [12], using bovine serum albumin as a standard.

Attachment assay

For attachment assays the cells were harvested, washed twice with growth medium and resuspended in the same medium at $2–4 \times 10^6$ cells/ml. Thirty-five-millimeter bacteriological plastic Petri dishes (Falcon Plastics, Cockeysville, MD) were coated with collagen as described previously [13]. The dishes were preincubated with 1 ml of growth medium at 37°C in 95% air–5% CO_2 for 1 hr. One hundred microliters of the cell suspension were then added to each dish and the incubation was continued for a further 3 hr. At the end of the incubation period the number of cells attached to each dish was determined as described for growth studies above. When the attachment assay was carried out in the presence of 2% DMSO or 1 mM butyrate the agent was present in the wash and in the incubation medium. The collagens used in the attachment assay were purified as described previously [14].

RESULTS

Effect of DMSO and butyrate on growth

The effects of a number of different concentrations of DMSO and butyrate on the growth of human pancreatic cancer cell lines were examined. To facilitate comparative studies the same experimental concentrations were selected for all the cell lines. The concentrations selected were the highest tested which did not cause significant cell death. A representative example of the changes which occurred when human pancreatic tumor

cells were grown in the presence of butyrate or DMSO is shown in Fig. 1. In this case, when MIA PaCa-2 cells were grown in 2% DMSO (Fig. 1A) the rate of growth of the cells was slowed down and the final density of cells on the plate was reduced. When the growth medium was changed to medium without DMSO, the growth rate began to change within a day or so and both the doubling time and the saturation density started to approach the values obtained for untreated cells. This demonstrated the reversibility of the effects of DMSO on the growth parameters of MIA PaCa-2 cells. When the same experiments were carried out using 1 mM butyrate a very similar set of growth curves was generated (Fig. 1B).

Growth experiments were also performed using the PANC-1 and CAPAN-1 cell lines, and the results obtained with all three cell lines are summarized in Table 1. Although the three cell lines have quite different rates of growth, in all cases 2% DMSO or 1 mM butyrate brought about a marked increase in the doubling time for each cell line. DMSO and butyrate also reduced the saturation density of all the cell lines except for PANC-1 cells grown in 2% DMSO, where no change could be detected. The relative effectiveness of the two agents varied from cell line to cell line.

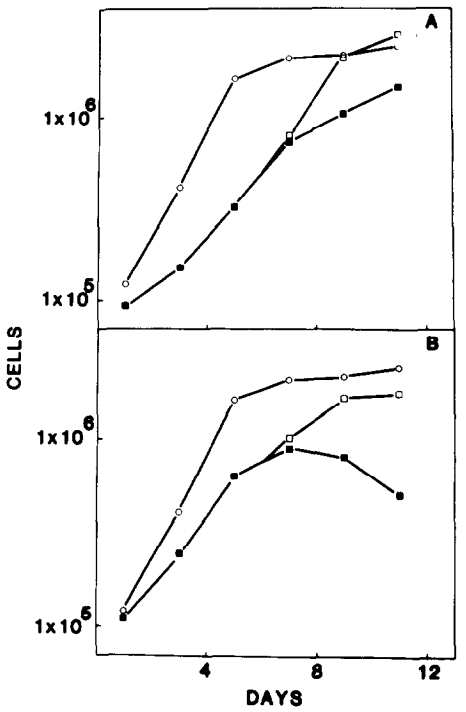

Fig. 1. The effect of DMSO (A) and sodium butyrate (B) on the growth of MIA PaCa-2 cells. 1 × 10⁵ cells were seeded in 35-mm Petri dishes in the absence or presence of 2% DMSO or 1 mM sodium butyrate. Duplicate dishes were harvested every 2 days and the number of cells in each dish was determined. Each point represents the mean of duplicate determinations. ○, Control medium; ■, cells grown in differentiating agent; □, cells grown in differentiating agent and changed to control medium on day 6.

Growth in soft agar

When the efficiency of colony formation in soft agar was determined, it was found that all three cell lines would form colonies in 0.3% agar with an inoculum of 5×10^4 cells (Table 2). MIA PaCa-2 cells had the highest colony-forming efficiency (27%), while PANC-1 and CAPAN-1 cells formed colonies with a lower efficiency (6 and 8.2% respectively). When 2% DMSO or 1 mM butyrate was present throughout the growth period, no colonies could be observed in the soft agar with any of the cell lines. Because of the slower growth rates of the cells in monolayer culture in DMSO or butyrate all colony counting was carried out at 16 and 28 days, but no significant difference was found between the results obtained at either time point. Similar results were obtained when the cells were inoculated at 5×10^3 cells per dish. Cells grown in DMSO or butyrate form colonies with a

Table 1. Effects of DMSO and butyrate on growth parameters of human pancreatic cancer cell lines

Cell line	Growth medium additions	Doubling time (hr)	Saturation density (cells/cm²)
MIA PaCa-2	none	26	2.3×10^5
MIA PaCa-2	DMSO	41	1.3×10^5
MIA PaCa-2	butyrate	38	9.6×10^4
PANC-1	none	38	2.9×10^5
PANC-1	DMSO	53	1.9×10^5
PANC-1	butyrate	60	7.8×10^4
CAPAN-1	none	41	1.6×10^5
CAPAN-1	DMSO	101	4.0×10^4
CAPAN-1	butyrate	82	6.8×10^4

Cells were grown on 35-mm Petri dishes, harvested at daily intervals and counted. The parameters were determined from the growth curves plotted on semi-logarithmic paper.

Table 2. Effects of DMSO and butyrate on growth in soft agar

Cell line	Colony-forming efficiency (%) None*	DMSO*	Butyrate*
MIA PaCa-2	27.00 ± 1.69†	<0.002	<0.002
PANC-1	6.00 ± 0.75	<0.002	<0.002
CAPAN-1	8.20 ± 2.65	<0.002	<0.002

5×10^4 cells were suspended in 0.3% agar over a layer of 0.5% agar in 35-mm Petri dishes. The numbers of colonies formed were counted after 16 and 28 days incubation.
*Additions to growth medium in agar.
†Mean ± S.E.

similar efficiency as untreated cells when placed in soft agar in the absence of the agent.

Enzyme assays

Human pancreatic tumor cell lines grown in the absence or presence of 2% DMSO or 1 mM butyrate were tested to determine if other cellular changes accompanied the inhibition of anchorage-independent growth by these agents. Amylase assays were carried out on sonicated samples of control and treated cells. In the samples tested no activity could be detected above background levels.

The levels of alkaline phosphatase were measured in sonicated samples of treated and untreated cells and the results obtained are shown in Table 3. Control human pancreatic tumor cell lines exhibit a wide range of cellular levels of alkaline phosphatase. MIA PaCa-2 cells have a very low level (0.46 IU/g protein), while CAPAN-1 cells have an approximately 200-fold higher cellular specific activity for alkaline phosphatase. When cells which had been grown in the presence of 2% DMSO were assayed for the same enzyme, significantly lower levels of the enzyme were found in PANC-1 and CAPAN-1 cells, although in the case of MIA PaCa-2 cells the already low level of the enzyme was not reduced further. Conversely, when cells which had been grown in 1 mM sodium butyrate were tested, it was found that the specific activity of alkaline phosphatase was increased in all three lines. The specific activity was increased only slightly in the CAPAN-1 cells, but was elevated about 20-fold in the other two lines. From these results it can be seen that DMSO and sodium butyrate have entirely opposite effects on the specific activity of alkaline phosphatase in human pancreatic tumor cell lines. The changes in specific activity are not due to changes in the protein content of treated cells as preliminary experiments indicate that butyrate treatment increases the amount of protein per cell while DMSO has little effect or reduces it. Thus, if the activities were expressed on a per cell basis, the changes would be more marked.

Attachment assays

The effects of DMSO and butyrate on the attachment of human pancreatic tumor cells to collagen-coated dishes were also measured. The results obtained are shown in Fig. 2A–C. In this case the cells were not grown in DMSO or butyrate prior to the attachment assay as there was no difference found between these results and those produced by cells grown in the agent prior to the assay. With all three cell lines, 1 mM butyrate had no effect on the attachment of the cells to type I or IV collagens after 3 hr. However, in the presence of 2% DMSO all the cell lines showed a large reduction in the attachment of the cells to both collagen types. In all three cases there was no attachment to uncoated plates in the presence or absence of the agents. For the sake of simplicity,

Table 3. Effects of DMSO and butyrate on cellular alkaline phosphatase activity

Cell line	Alkaline phosphatase activity (IU/g protein) None*	DMSO*	Butyrate*
MIA PaCa-2		0.46 ± 0.11	9.41 ± 0.09
PANC-1	3.47 ± 0.09	1.07 ± 0.10	81.50 ± 1.25
CAPAN-1	81.75 ± 4.09	3.55 ± 0.05	99.00 ± 2.41

Cells grown in the absence or presence of DMSO or butyrate were washed, harvested and sonicated. The specific activity of alkaline phosphatase in the sonicates was measured.
*Additions to growth medium.
†Mean ± S.E.

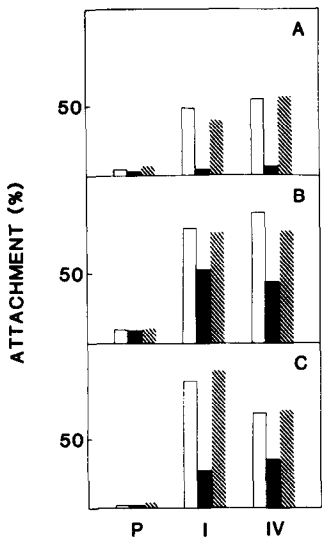

Fig. 2. The effect of DMSO and sodium butyrate on the attachment of MIA PaCa-2 (A), PANC-1 (B) and CAPAN-1 (C) cells to type I and IV collagens and to uncoated plastic dishes (P). The attachment assays were carried out as described in Materials and Methods in the absence or presence of 2% DMSO or 1 mM sodium butyrate. Open bar—no addition; black bar—DMSO; shaded bar—sodium butyrate.

the attachment to type I and IV collagens is shown, but similar results were obtained when type II, III and V collagens were used in the attachment assay. One explanation for the effect of DMSO would be if, during the pre-incubation period, the 2% DMSO were solubilizing a portion of the collagen coated on the plate. However, control experiments proved that this was not the case.

DISCUSSION

The results presented in this report describe the effects of DMSO and sodium butyrate on human pancreatic tumor cell lines. Three different cell lines and two different agents were used in an effort to avoid any cell-line-specific effects. In the case of pancreatic tumor cells it will be more difficult to relate changes in the tumorigenicity of the cells with alterations in the degree of differentiation of the cells as no known specific markers exist for the pancreatic ductal epithelial cells from which most pancreatic carcinomas are thought to be derived [8]. Indeed, the approach used in this study could lead to the development of differentiation markers for such cells. At present, however, the main aim of this investigation is the correlation between the reduction of parameters associated with tumorigenicity of the human pancreatic cancer cells and changes in other cellular characteristics.

The first step is to establish an effect of DMSO or sodium butyrate on the parameters correlated with tumorigenicity of the cell lines studied. When the cells are grown in monolayer cultures in the presence of DMSO or butyrate the doubling times of the cell lines are increased and the saturation densities are reduced. A similar effect has been observed when other cell lines are grown in the presence of these differentiating agents [4, 6]. These changes in the growth characteristics of the human pancreatic tumor cell lines may reflect a less tumorigenic phenotype being expressed in the presence of DMSO or butyrate. In studies with adult rat liver epithelial-like cell lines, non-tumorigenic cells had a lower saturation density and longer doubling time than their tumorigenic counterparts in many cases [15]. In the case of the human pancreatic cancer cell lines, the decreased saturation density is not due to any morphological change in the cells visible under light microscopy. It might be related to a decrease in the ability of the cells to overgrow one another, but this is difficult to quantitate.

Although the growth characteristics of treated human pancreatic tumor cells suggest a reduced tumorigenicity, the *in vitro* property which correlates best with tumorigenicity is the ability of the cells to form colonies in soft agar [16, 17]. When the cell lines were tested for growth in soft agar in the presence and absence of butyrate or DMSO it was found that both agents inhibited the anchorage-independent growth of all three cell lines. Thus both 1 mM butyrate or 2% DMSO will reversibly alter the phenotype of human pancreatic cell lines to that associated with much less tumorigenic cells.

In an effort to elucidate the molecular changes involved in this alteration, several properties of the treated and untreated cells were compared. First, because of the common embryological origin of the ductal and acinar cells in the pancreas, the levels of amylase, a marker for acinar cells, were determined in treated and untreated cells. The lack of activity in all cases indicates that none of the cell lines are assuming acinar cell characteristics in the presence of the differentiating agents.

The specific activity of alkaline phosphatase in treated and untreated cells was also examined. This enzyme was measured for several reasons. Although it is found in human pancreatic carcinoma cells [18], it appears to be localized in the connective tissue of normal pancreatic ducts, not in the epithelial cells [10]. Elevated levels of alkaline phosphatase have been found in human colon tumors [19], but the activity of the enzyme in transformed cells is reduced relative to normal cells [20]. Thus the correlation between levels of the enzyme and the malignant state is, as yet, uncertain. The results obtained in this study did not alter this uncertainty as butyrate increased the

specific activity in all three cell lines while DMSO caused a decrease in two lines (PANC-1 and CAPAN-1) and no change in the third. The results with DMSO suggest that the agent reduces the level of cellular alkaline phosphatase to a basal level but will not reduce it further. The elevation of the level of cellular alkaline phosphatase by butyrate has been demonstrated in other cell lines [21].

The effects of DMSO and butyrate on the attachment of human pancreatic cancer cells to immobilized collagen were measured. The interaction of a cell with the extracellular matrix is an important event in tumorigenicity since it is the basis of invasive and metastatic processes. In this case, also, the two agents had different effects: butyrate did not change the attachment while DMSO greatly reduced it in all cases. This may provide a useful tool to probe the molecules involved in attachment. It is significant that the effect of DMSO on attachment can be seen after 3 hr exposure to the agent and may be detectable even earlier. This is one of the earliest changes that has been measured in studies of the effects of differentiating agents [2], although most of the experiments were performed on erythroleukemic cells. The detection of early events in the sequence of changes produced by differentiating agents is of great importance in understanding the mechanism and action of these molecules.

The fact that DMSO and butyrate have markedly different effects on the cell properties of human cancer cells has also been noted by other investigators. Kim *et al.* [4] have shown that DMSO and butyrate alter the cell-surface properties of human colon cancer cells in different ways. Dexter *et al.* [23] found that *N,N*-dimethylformamide, which is similar to DMSO in its action, had an effect on the purine-metabolizing enzymes of human colon cancer cells which varied from that of sodium butyrate. Other studies have classified DMSO and butyrate in two separate groups of inducers of differentiation in erythroleukemic cells [24, 25]. This study suggests that such a classification can be applied to the action of the inducers on human tumor cell lines.

The results reported here establish a model system to examine the properties of human pancreatic cancer cells which are related to the tumorigenicity of the cells. In the cellular characteristics studied so far, no common change has been brought about by DMSO and butyrate. It is entirely possible for a phenotypic change to be achieved by more than one mechanism [22], and the effects of DMSO and butyrate may both have a bearing on the molecules involved in tumorigenicity. Their modes of action will be further elucidated as this study is extended to encompass other characteristics of human pancreatic cancer cell lines.

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