Emergence of Differentiated Subclones from a Human Salivary Adenocarcinoma Cell Clone in Culture after Treatment with Sodium Butyrate

Masayuki Azuma, Yoshio Hayashi, Hideo Yoshida, Tetsuo Yanagawa, Yoshiaki Yura, Akemichi Ueno, and Mitsunobu Sato

Second Department of Oral and Maxillofacial Surgery [M. A., H. Y., T. Y., Y. Y., M. S.], Central Laboratory for Clinical Investigation [Y. H., J], and Department of Biochemistry [A. U.], Tokushima University School of Dentistry, 3 Kuramoto-cho, Tokushima 770, Japan

ABSTRACT

A human salivary gland adenocarcinoma cell line, which has ultrastructure and biological markers specific to the intercalated duct cells of human salivary glands, was cultured in 5 mM sodium butyrate for 12 days; then the cells were trypsinized, subcultured for an additional 16 days, and then transferred to growth medium without sodium butyrate. Morphological changes appeared about 1 wk after return to growth medium without sodium butyrate; cells being spindle or stellate in shape appeared in the treated cells, whereas the untreated cells were polygonal in shape. This morphologically altered phenotype persists after more than 14 mo of culture in growth medium without sodium butyrate. Of 40 subclones isolated, 2 clonal cell lines were established from the subculture and characterized. The other 38 subclones were accompanied by cell death during the subcultures. The clonal lines exhibited a phenotype similar to myoepithelial cells such as myofilaments, β-chain of S-100 protein, and oxytocin receptor, and the cells have markedly decreased tumorigenicity as compared to that of their parental cells.

INTRODUCTION

There is increasing evidence that the intercalated duct cells present in human salivary glands can differentiate into myoepithelial cells on the basis of the morphological investigations on human salivary gland neoplasms (1–6). While establishment and characterization of a human salivary gland adenocarcinoma cell line having ultrastructure and biological markers specific to the intercalated duct cell of the salivary gland have been reported by our study group (7–10), we have recently found that reversible differentiation into myoepithelial cells such as myosin, β-chain of S-100 protein, myofilaments, and oxytocin receptor in addition to decreased tumorigenicity and anchorage-independent growth. These findings indicate that commitment to differentiation into myoepithelial cells and conversion from malignant to normal phenotype occur in a human salivary gland adenocarcinoma cell line following the treatment with sodium butyrate.

MATERIALS AND METHODS

Culture and Media. The human salivary gland adenocarcinoma cell line, HSG, was grown in Eagle’s MEM supplemented with 10% newborn calf serum and 2 mM L-glutamine in the presence of 5% CO2 in an incubator at 37°C. Isolation and characterization of HSG cells were described previously (7–10).

When the HSG cells accomplished 485 cell generations from the start of the culture, clonal cell lines were cloned a second time using the colony-forming technique in semisolid agar medium, as described previously (7, 9). Briefly suspensions of 102 or 103 cells in a growth medium containing 0.3% Noble special agar (Difco Laboratories, Detroit, MI) were poured on the agar medium which had been hardened by addition of 0.6% agar into the growth medium. The colonies that formed after 20 days of incubation at 37°C in a 5% CO2 incubator were isolated with Pasteur’s pipets, and the cells were cultured in 3 ml of growth medium in 35-mm plastic Petri dishes (Falcon Co., Oxnard, CA) until the formation of confluent cell monolayers. The growth medium was changed twice a week during that period. Of 10 colonies isolated, the one clone which showed the most stable growth was used for the current study. This HSG clone had an ultrastructure specific to the intercalated duct cells of the human salivary gland; expressed the biological markers such as carcinoembryonic antigen, secretory component, and lactoferrin; and formed an adenocarcinoma upon transplantation of the cells into nude mice, as previously described in the parental HSG cells (7, 8).

Treatment of HSG Clone with Sodium Butyrate. The monolayer cultured cells of the HSG clone were dissociated by treatment with...
0.08% (w/v) trypsin and 1.4% (w/v) EDTA in calcium- and magnesium-free PBS (100 mM sodium phosphate:120 mM NaCl, pH 7.2), and then 10³ cells/dish were seeded in 60-mm plastic Petri dishes (Falcon) at Day 0 in growth medium. At Day 1, sodium butyrate (Wako Pure Chemical Co., Osaka, Japan) was added at a concentration of 5 mM. Then the growth medium containing sodium butyrate was changed every 3 days. The cells were dissociated with trypsin solution at Day 12, and then after a 1:3 split, the cells were plated in 60-mm plastic Petri dishes in growth medium containing sodium butyrate (5 mM). Then the medium containing sodium butyrate was changed every 2 days. At Day 28, the cells were again trypsinized as described above and plated in fresh growth medium without sodium butyrate in 60-mm plastic Petri dishes. The cells were then routinely subcultured in the growth medium without sodium butyrate at 3- to 4-day intervals.

Cell Cloning. Some clonal lines were subconfluent using a dilution plating technique. Briefly a single-cell suspension was diluted appropriately in growth medium containing 20% newborn calf serum to yield a final number of 30 cells/ml. This cell suspension was dispensed into the wells of microtest plates (96-well microtest plates; Falcon). Each well was inoculated with 50 μl of the cell suspension, yielding on the average 1.5 cells/well. Those wells containing only one cell were ascertained by microscopic inspection. The plates were then incubated at 37°C, and the cells from selected wells were subcultured into separate 35-mm culture dishes. All of these clonal lines were subcultured and kept frozen in liquid nitrogen at an early passage.

Plating Efficiency. To study the plating efficiency of cells on a plastic surface, cells were dispensed into a single-cell suspension with trypsin solution and were plated into a 60-mm plastic Petri dish at a density of 10² or 10³ cells/5 ml of growth medium. They were incubated for 10 days at 37°C in a 5% CO₂ incubator. After being stained with 10% Giemsa solution, the colonies formed were macroscopically counted. The plating efficiency of cells in semisolid agar medium was studied using the colony-forming technique, as described above.

Immunoperoxidase Staining. The immunohistochemical study of the cultured cells was performed according to the PAP method described by Sternberger et al. (12). The specific antigens chosen were myosin, α- and β-chains of S-100 protein, and CEA. The cultured cells were washed 3 times with PBS and fixed with a mixture of 95% ethanol and acetone at a 4:5 ratio for 10 min at 4°C. They were incubated at 37°C with 0.3% H₂O₂ in absolute methanol for 10 min to block endogenous peroxidase. After being washed 3 times with PBS, they were incubated with an appropriate dilution of rabbit antiserum to myosin or CEA, or of mouse monoclonal antibody to α- or β-chain of S-100 protein, for 30 min at room temperature followed by a rinse with PBS for 30 min. Thereafter, goat anti-rabbit IgG or goat anti-mouse IgG (Medical and Biological Laboratory, Ltd., Nagoya, Japan) was applied in a dilution of 1:40 in PBS for 30 min at room temperature. After being washed with PBS for 30 min, these samples were incubated with rabbit horseradish PAP complex (Miles Laboratories, Inc., Elkhart, IN) or mouse PAP complex (Jackson ImmunoResearch Laboratories, Inc., Avondale, PA), diluted 1:50 in PBS for 30 min, and rinsed with PBS for 20 min. Finally the peroxidase was localized by treatment of the samples with a fresh mixture of 0.05% 3′,3′-diaminobenzidine and 0.01% H₂O₂ in 50 mM Tris-HCI buffer (pH 7.6) for 5 min, and after being washed with distilled water, these samples were counterstained with hematoxylin.

The following controls were used. The rabbit antiserum raised against CEA, which was used in the present study, was absorbed with normal human spleen extract to remove nonspecific cross-reacting antigen as follows. Antibody sample (0.2 ml) was mixed with 100 μg of acetone powder, prepared from normal human spleen according to the conventional method, and was incubated at room temperature for 2 h. Thereafter, 0.8 ml of PBS was added to this mixture, and the supernatant obtained by centrifuging at 500 x g for 15 min at 4°C was filtered through a Millipore membrane, type HA (Millipore Co., San Francisco, CA), and used as an absorbed antiserum for the present study. A blocking test in the use of anti-CEA serum was provided by incubating parallel samples with the anti-CEA serum absorbed with CEA extract of human colon cancer tissues. One-fifth ml of the rabbit antiserum raised against myosin or of mouse monoclonal antibody to α- or β-chain of S-100 protein was added to 0.8 ml of PBS, mixed with 200 μg of chicken myosin or S-100 protein from bovine brain, and incubated at room temperature for 2 h. Thereafter, they were centrifuged at 500 x g for 15 min at 4°C and then filtered through a Millipore membrane, type HA (Millipore Co.) to remove microaggregates. The supernatants were used as absorbed antisera for the present study. These antibody samples gave negative results. Myosin from muscle was purchased from Sigma Chemical Co., St. Louis, MO, and S-100 protein from bovine brain was purchased from Calbiochem-Behring, Inc., La Jolla, CA. Negative controls were provided by incubations of parallel samples with a relevant dilution of normal rabbit serum or of normal mouse serum instead of the specific antibodies of the initial incubation.

The rabbit antiserum to chicken myosin was purchased from Transformation Research, Inc., Framingham, MA. The rabbit antisera to CEA was purchased from Dakopatts, Copenhagen, Denmark, and mouse monoclonal antibody to α- or β-chain of S-100 protein was purchased from Otsuka Assay Laboratory, Inc., Tokushima, Japan. In the experiments, anti-CEA serum was used at a dilution of 1:1000, antmyosin serum was used at a dilution of 1:100, and mouse monoclonal antibody to α- or β-chain of S-100 protein was used at a dilution of 1:5.

Morphological Observation. The monolayer cell culture was fixed in methanol and stained with 0.1 M phosphate-buffered Giemsa (pH 6.8). For transmission electron microscopy, the cultured cells harvested by scraping with a rubber policeman were fixed in 2.5% glutaraldehyde buffered with 0.1 M phosphate (pH 7.4) containing 0.005 M calcium chloride for 2 h and then dissected into small pieces of 2 mm³. After washing in the phosphate buffer, these materials were postfixed with 1% osmium tetroxide, dehydrated step by step with ethanol, and embedded in Epon 812. The ultrathin sections were cut on an LKB ultramicrotome and stained with uranyl acetate and lead hydroxide. The finished preparations were observed under a Hitachi Model H-500 electron microscope.

For immunoelectron microscopy, the cell monolayers were washed in PBS and fixed with peroxidase/livine/parafomaldehyde as described by Mclean and Nakane (13) for 2 h at 4°C followed by washing for 1 h in 5 changes of PBS containing 10% (w/v) sucrose. Thereafter, cells were incubated overnight with antmyosin rabbit serum at 4°C. After being washed in PBS for 30 min, cells were reacted with horseradish peroxidase-labeled Fab’ fragment of anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) for 5 h at room temperature. After being washed in PBS for 30 min, these preparations were fixed in 2% glutaraldehyde for 30 min at 4°C and then washed 3 times in PBS. Then they were incubated for 5 min in Karnovsky’s solution containing 0.1% dimethylaminobenzidine (Wako Pure Chemical Co.) and 0.03% hydrogen peroxide (Wako) in 50 mM Tris-HCl buffer (pH 7.6) at room temperature and then washed in PBS. After being incubated in 2% osmium tetroxide for 1 h at 4°C, they were dehydrated step by step with ethanol and embedded in Epon 812 resin. After polymerization, the Epon sheet was detached from the blocks. Ultrathin sections were cut on an LKB ultramicrotome and examined without staining under a Hitachi electron microscope. A control experiment was done with normal rabbit serum.

Autoradiography. The oxytocin receptor in the cells was examined by the autoradiographic method as described by Baserga (14).

The cells were grown in a Bellco microliter culture chamber (0.317 cm²/hole) (Bellco Glass, Inc., Vineland, N.J.) in a 5% CO₂ incubator at 37°C for 2 days. The formed monolayers were washed with MEM and incubated with 125I-oxytocin (specific activity, 2200 Ci/mM; New England Nuclear, Boston, MA) in various concentrations of MEM ranging from 10 nCi/ml to 1 pCi/ml at 37°C for 1 h. Thereafter, the cells were washed with PBS and fixed in 0.1 M phosphate-buffered 10% formalin for 10 min, followed by washing in running tap water for about 3 h. Then the
cell samples were dip coated in Sakura autoradiographic emulsion, type NR-M2 (Konishiroku Photo Industrial Co., Ltd., Tokyo, Japan), dried, and exposed to NR-M2 in the dark room for 2 wk. Thereafter, these samples were developed by Copinal (Fuji Photo Films Co., Tokyo, Japan) and then were stained with hematoxylin:eosin.

Immunoblotting. The crude preparation of myosin from freshly harvested cells was performed according to the method of Clarke and Spudich (15). Briefly the cells (2 × 10^6) were homogenized in 10 mM Tris-HCl buffer (pH 7.9) containing 1 mM EDTA, 0.1 mM dithiothreitol, 30% (w/v) sucrose, and 0.1 mM KCl. After centrifuging the homogenate at 23,000 × g for 30 min, the supernatant fluid was collected and dialyzed overnight against the Tris-HCl buffer containing 0.1 mM KCl. Then the cell extract was centrifuged at 23,000 × g for 30 min to collect the actomyosin. This pellet was suspended in the Tris-HCl buffer containing 0.61 M KCl, 10 mM ATP, 10 mM MgCl2, and 2 mM CaCl2. After homogenizing gently and then centrifuging at 100,000 × g for 30 min, the supernatant fluid was applied immediately to the SDS-PAGE.

The procedure for SDS-PAGE used in the present study essentially followed the method of Laemmli (16). The slab gels measured 8.0 × 12.0 × 0.2 cm. The stacking gel contained 5% acrylamide (Wako) and 0.13% N,N'-methylene bisacrylamide. The electrophoresis was performed at room temperature for 4 h at a current of 4 mA/cm of gel in width. The migration front was followed by adding 0.002% bromophenol blue to the electrode buffer. The gel was stained for 12 h, using 0.25% Coomassie brilliant blue in 35% ethanol:10% acetic acid in water, and destained in the same solution minus the stain. Proteins from the gels were transferred to nitrocellulose paper as reported by Towbin et al. (17). Detection of myosin on strips was performed by immune autoradiography according to the method of Burnette (18). Briefly the nitrocellulose sheets were incubated with an appropriate dilution of antitumor serum in 0.5 M Tris-HCl buffer (pH 6.8) containing 5% (w/v) bovine serum albumin (Sigma Chemical Co.) at room temperature for 90 min. After being washed in the Tris-HCl buffer with or without 0.05% (v/v) Nonidet P-40 (Nakarai Chemical Ltd., Tokyo, Japan), the nitrocellulose sheets were reacted with 125I-Protein A (5 × 10^6 cpm/ml) (specific activity, 8.4 μCi/μg; New England Nuclear) in 0.5 M Tris-HCl buffer (pH 6.8) containing 5% (w/v) bovine serum albumin at room temperature for 30 min. Thereafter, the nitrocellulose sheets were washed with the Tris-HCl buffer and then exposed to X-ray films (Kodak X-Omat RP; Eastman Kodak Co., Rochester, NY).

Tumorigenicity. For the purpose of the present study, 6-wk-old female BALB/c nude mice were purchased from Clea Japan, Inc., Tokyo, Japan. All mice were maintained in a pathogen-free environment in a vinyl isolator (Clea Japan).

Mice received a total number of 10^7 or 4 × 10^6 cells s.c. through an injection needle in the anterior aspect of the lateral thoracic region. Tumor growth was observed weekly, and animals were sacrificed by cervical dislocation at 1 to 3 mo after the inoculation of cells. Tumors were fixed in 0.1 M phosphate-buffered 10% formalin.

RESULTS

Effect of Sodium Butyrate on HSG Clone. During sodium butyrate treatment, major morphological alteration, except cell rounding which appeared sporadically in the treated HSG monolayers, did not occur. At this time, the presence of CEA was detected immunohistochemically in almost all of the cells. About 1 wk later following removal of sodium butyrate from the culture, major morphological alteration occurred; cells which were spindle or stellate in shape began to appear in the treated HSG cells, whereas the untreated cells were polygonal in shape and grew in the tendency of the formation of cell clusters (Fig. 1). After an additional 1 wk, almost 100% of the population of cells exhibited this altered morphology. About 3 mo after removal of sodium butyrate from the culture, we attempted to isolate by a dilution plating technique subclones from the subculture of the treated HSG cells in which morphological change and cell growth were maintained. Of 40 subclones isolated, 2 subclones alone could be expanded in monolayer culture. These subclones were designated as HSG-BT1 and HSG-BT2 and were used for the following experiments. Although other subclones were transiently grown, their subcultures resulted in cessation of growth, and the cells could not be maintained in monolayer culture.

Growth Characteristics of HSG-BT Subclones. The growth characteristics of HSG-BT cells are shown in Fig. 2. The respective doubling times for HSG-BT1 and HSG-BT2 cells were 95.8 and 93.5 h, while the doubling time for the parental HSG clone was 34.8 h. The cultured HSG-BT1 and HSG-BT2 cells at the second passaged level formed colonies in semisolid agar with efficiencies of 4.3% and 4.8%, respectively, while colony-forming ability of the parental HSG clone in semisolid agar was 26.7% (Table 1). The percentage of colony formation on a plastic surface was 53.4% in HSG-BT1 cells, 61.7% in HSG-BT2 cells, and 94.1% in the parental HSG cells.

When a total of 10^7 HSG-BT1 or HSG-BT2 cells were transplanted into SCID mice and allowed to grow, tumors, which were characterized by their large size and hard consistency, were obtained. The mice died of tumor cachexia 7 mo after transplantation of the HSG-BT1 cells (91% tumor incidence). In contrast, the mice inoculated with HSG-BT2 cells (93% tumor incidence) lived longer than the mice inoculated with HSG-BT1 cells and died of tumor cachexia 10 mo after transplantation of the HSG-BT2 cells. Two samples of subclones were tested for tumor incidence in NSG mice and BALB/c nude mice, respectively. In the NSG mice, the tumor incidence was 50% for HSG-BT1 but not for HSG-BT2. In contrast, tumor incidence was 100% for HSG-BT1 but not for HSG-BT2 in BALB/c nude mice.
DIFFERENTIATION OF A SALIVARY ADENOCARCINOMA CELL CLONE

Fig. 2. Growth curves of 2 subclones isolated from the morphologically altered HSG clone. O, HSG-BT1 subclone; △, HSG-BT2 subclone; ◀, parental HSG clone. Cells were plated in 35-mm plastic Petri dishes at a density of 5 x 10^5 cells/dish and incubated at 37°C in a 5% CO_2 incubator. The numbers of viable cells as a function of time were counted by a hemocytometer using the trypsin blue exclusion test. For the sake of clarity, each point represents an average of 4 samples.

Table 1
Colony-forming ability of HSG-BT subclones and their parental HSG clone

<table>
<thead>
<tr>
<th>Cell</th>
<th>No. of cells</th>
<th>No. of colonies in semisolid agar</th>
<th>No. of colonies on plastic surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSG-BT1</td>
<td>1 x 10^5</td>
<td>5, 3</td>
<td>54, 68</td>
</tr>
<tr>
<td></td>
<td>1 x 10^6</td>
<td>49, 37</td>
<td>592, 535</td>
</tr>
<tr>
<td>HSG-BT2</td>
<td>1 x 10^5</td>
<td>6, 5</td>
<td>58, 71</td>
</tr>
<tr>
<td></td>
<td>1 x 10^6</td>
<td>51, 44</td>
<td>605, 629</td>
</tr>
<tr>
<td>Parental HSG</td>
<td>1 x 10^5</td>
<td>37, 28</td>
<td>88, 92</td>
</tr>
<tr>
<td>clone</td>
<td>1 x 10^6</td>
<td>275, 258</td>
<td>936, 943</td>
</tr>
</tbody>
</table>

a Cells were mixed with 1 ml of 0.3% agar medium and then overlaid onto a basal layer which had been made by pouring 2 ml of 0.6% agar medium into a 35-mm plastic Petri dish. After 20 days of incubation at 37°C, the colonies formed were counted.

b The singly dispersed cells (10^5 or 10^6) were plated in a 60-mm plastic Petri dish and then cultured for 10 days at 37°C in a 5% CO_2 incubator. After staining with Giemsa solution, visible colonies formed were counted.

Table 2
Decreased tumorigenicity of HSG-BT subclones as compared to that of the parental HSG clone

<table>
<thead>
<tr>
<th>Cell</th>
<th>No. of cells inoculated</th>
<th>No. of mice which developed tumor/no. of mice examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSG-BT1</td>
<td>1 x 10^7</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>4 x 10^7</td>
<td>2/10</td>
</tr>
<tr>
<td>HSG-BT2</td>
<td>1 x 10^7</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>4 x 10^7</td>
<td>3/10</td>
</tr>
<tr>
<td>Parental HSG</td>
<td>1 x 10^7</td>
<td>5/5</td>
</tr>
<tr>
<td>clone</td>
<td>4 x 10^7</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

Biological Markers and Ultrastructure of HSG-BT Subclones. We examined immunohistochemically the expression of specific antigens such as myosin, α- and β-chains of S-100 protein, and CEA in the HSG-BT subclones cultured for 4 days at 37°C. Fig. 4 shows a representative sample of the immunohistochemical appearance. Table 3 summarizes all of the results obtained by the immunohistochemical study. The presence of myosin and β-chain of S-100 protein in HSG-BT subclones was clearly observed, whereas the untreated parental HSG cells did not express these antigens. Almost all of the HSG-BT subclone population showed positive staining for myosin and β-chain of S-100 protein. However, CEA observed in the parental HSG cells disappeared in cultured HSG-BT subclones. The presence of α-chain of S-100 protein was not detected in either HSG-BT or parental HSG cells. In addition, the expression of oxytocin re-
Fig. 4. Immunohistochemical search for myosin, α- or β-chain of S-100 protein, and CEA in the cultured HSG-BT1 cells. a, antimyosin (positive); b, anti-β-chain of S-100 protein (positive); c, anti-α-chain of S-100 protein (negative); d, anti-CEA (negative); e, absorbed antimyosin (negative); f, absorbed anti-β-chain of S-100 protein (negative). Antisera used in e and f were absorbed with each corresponding antigen. Anti-α-chain of S-100 protein stained the frozen tissue section from human brain (g), and anti-CEA stained the cultured HSG cells (h). Hematoxylin counterstain, a to h; ×720. Cells were cultured in the absence of sodium butyrate for 4 days at 37°C and then were examined for the specific antigens. The cultured HSG-BT2 cells showed positive staining for the above specific antigens in a fashion similar to that observed in the cultured HSG-BT1 cells (data not shown).
DIFFERENTIATION OF A SALIVARY ADENOCARCINOMA CELL CLONE

Table 3
Summary of the immunohistochemical characteristics of cultured HSG-BT subclones and of their parental HSG clone

<table>
<thead>
<tr>
<th>Specific antigens</th>
<th>HSG-BT1</th>
<th>HSG-BT2</th>
<th>Parental HSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin</td>
<td>96.5</td>
<td>95.0</td>
<td>0</td>
</tr>
<tr>
<td>α-Chain of S-100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>β-Chain of S-100</td>
<td>95.5</td>
<td>97.5</td>
<td>0</td>
</tr>
<tr>
<td>CEA</td>
<td>0</td>
<td>0</td>
<td>95.5</td>
</tr>
</tbody>
</table>

% of positive cells

DISCUSSION

This paper reports the emergence of cells having the phenotype of myoepithelial cells in a human salivary gland adenocarcinoma cell line HSG in culture after treatment with sodium butyrate; e.g., the morphologically altered cells which are cultured in the absence of sodium butyrate express consistently the biological markers, such as myosin, probably S-100b protein being composed of β-chains (19, 20), myofilaments, and oxytocin receptor, which are specific to the myoepithelial cells of human salivary glands as indicated in other published papers including ours (7–9, 11, 21). In addition, these altered cells were found to carry decreased tumorigenicity and anchorage-independent growth potential as compared to those of their parental cells.

It has been reported that sodium butyrate produces reversible changes in morphology, growth rate, and enzyme activities of several cell types in culture (22). The addition of sodium butyrate to several human colonic and rectal cancer cells in culture has been shown to cause a decrease of the growth rate, morphological changes, and an increase of some enzyme activities, such as alkaline phosphatase and sucrase, although all of these changes were found to be reversible (23–26). In addition, Tsao et al. (27) have reported that treatment of some human colorectal cell lines with sodium butyrate results in an elevation of CEA.

Fig. 5. Demonstration of oxytocin receptors on HSG-BT1 subclone by autoradiography. Photomicrograph demonstrating the presence in HSG-BT1 cells of many grains which show the binding of [125I]-oxytocin with its receptors in the cells.

Fig. 6. Immunoblotting of HSG-BT myosin with antibodies to chicken myosin. The myosin preparation was made from the cultured cells of HSG-BT subclones according to the method of Clarke and Spudich (15) and was run on a slab gel. After the protein was transferred to nitrocellulose, the blot was cut into strips and incubated with rabbit antisera specific to chicken myosin. Lane a, chicken myosin (Sigma Chemical Co.); Lane b, myosin preparation from HSG-BT1 subclone; Lane c, myosin preparation from HSG-BT2 subclone; Lane d, myosin preparation from parental HSG clone. Ordinate, molecular weight markers x 103. Ferritin (half unit), M, 220,000; phosphorylase b, M, 94,000; bovine serum albumin, M, 67,000; ovalbumin, M, 43,000; carbonic anhydrase, M, 30,000; soybean trypsin inhibitor, M, 20,000; α-lactoalbumin, M, 14,400.
content in the cells and that sodium butyrate may enhance expression of the differentiated function of the cells.

The effects of sodium butyrate on HSG cells can be explained by the creation of irreversible modifications in the cells but not by a selection mechanism, because the HSG cells used for the current study are subjected to cell cloning before the sodium butyrate treatment. In addition, it is well known that sodium butyrate induces hyperacetylation of histones mainly as a consequence of inhibition of histone deacetylase (28), and it has been suggested that this process is implicated in the control of gene expression. Although the morphologically altered cells that appeared in the HSG clone following the sodium butyrate treatment were not observed in cultured cells of the HSG clone without sodium butyrate treatment, the possibility that the HSG clone may become heterogeneous during the process of expansion in monolayered culture before the sodium butyrate treat-

---

**Fig. 7.** Electron micrographs of cultured HSG-BT1 subclone. a, cell with oval nucleus containing numerous microfibrils and mitochondria. \( \times 6,000 \). b, high power view of a inset showing bundles of fibrils running parallel to the cytoplasmic membrane. \( \times 16,000 \).

**Fig. 8.** Detection of myofilaments in cultured HSG-BT1 subclone. Immunoelectron micrograph without staining by uranyl acetate and lead hydroxide, demonstrating the presence of microfilamentary systems reactive to antimyosin serum in the cultured HSG-BT1 subclone. \( \times 15,000 \).
DIFFERENTIATION OF A SALIVARY ADENOCARCINOMA CELL CLONE

ment and, as a consequence, some cells spontaneously achieving a high level of differentiation may be selected by sodium butyrate treatment is not neglected. Augeron and Laboisse (29) have recently reported that permanently differentiated cell clones emerge in a human colonic cancer cell line in culture after treatment with sodium butyrate.

Although 40 subclones were isolated from the irreversibly altered cells which emerged following sodium butyrate treatment of the HSG clone, only 2 subclones could be sequentially subcultured, and the other 38 subclones could not be maintained in culture. In addition, the surviving 2 subclones showed markedly decreased tumorigenicity and anchorage-independent growth as compared to those of their parental clone. When a terminally differentiated phenotype is induced in a culture such as a human promyelocytic leukemia HL-60 cell line or Friend leukemia cells, the reconstitution of the population of self-renewing cells from the uninduced cells proceeds in the absence of inducer and is accompanied by the death of the differentiated postreplicative cells (30, 31). In the current study, it can be considered that the HSG cells which were subcultured in growth medium without sodium butyrate following the sodium butyrate treatment differentiated into myoepithelial cells of which most cells represented the phenotype of normal cells, resulting in a failure to obtain many subclones which showed sequentially stable growth. On the other hand, it seems that the isolated 2 subclones are blocked in their differentiation at some step preceding a terminal differentiation, and, for this reason, they retain tumorigenicity and can be sequentially subcultured because they do not yet completely represent the phenotype of normal myoepithelial cells.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Nanayo Furimoto for secretarial assistance.

REFERENCES


3. Hubner, G., Kleinasser, O., and Klein, H. J. Zur Finestruktur und Genese der Cyldrome der Speiechdrusen. Weitere Untersuchungen zur Rolle myoepithe-


5. Batsakis, J. G. Salivary gland neoplasia: an outcome of modified morpho-


10. Hayashi, Y., Yanagawa, T., Yoshida, H., Yura, Y., Nitta, T., and Sato, M. Induction of other differentiation stages in neoplastic epithelial duct and myo-


17. Towbin, H., Staehelin, T., and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some appli-


19. Isobe, T., Ishioka, N., Misuda, T., Takahashi, Y., Ganno, S., and Okuyama, T. A rapid separation of S-100 subunits by high performance liquid chromatog-


27. Taso, D., Monta, A., Bella, A. Jr., Luu, P., and Kim, Y. S. Differential effects of sodium butyrate, dimethyl sulfoxide, and retinoic acid on membrane-assoc-


CANCER RESEARCH VOL. 46 FEBRUARY 1986

777

Downloaded from cancerres.aacrjournals.org on April 20, 2017. © 1986 American Association for Cancer Research.
Emergence of Differentiated Subclones from a Human Salivary Adenocarcinoma Cell Clone in Culture after Treatment with Sodium Butyrate

Masayuki Azuma, Yoshio Hayashi, Hideo Yoshida, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/2/770

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.