Expression of Neurofilaments in a Neoplastic Human Salivary Intercalated Duct Cell Line or Its Derivatives and Effect of Nerve Growth Factor on the Cellular Proliferation and Phenotype

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ABSTRACT

A neoplastic salivary cell line with an ultrastructure similar to that of an intercalated duct cell of the salivary gland, established from a human submandibular salivary gland, has been used in our laboratory as a model for studying mechanisms regulating cytodifferentiation in salivary glands. The expression of neurofilaments (M, 200,000, 160,000, and 68,000) in the neoplastic human salivary intercalated duct cell line and its derivatives was found by the immunofluorescence staining technique, immunoblotting, or immuno electron microscopy. In addition, these cells stained with Bodian impregnation and expressed specific antigens such as tubulin α and β chain, HNK-1 antigen, and laminin. When these cells were cultured in the presence of nerve growth factor, only the cells with a myoepithelial cell phenotype formed the long cytoplasmic processes which were densely packed with ample microfibrils in addition to microtubule bundles, and they exhibited marked suppression of anchorage-independent and anchorage-dependent growths. These findings indicate that the characteristics of neoplastic human salivary intercalated duct cell line and its derivatives are similar to those of neuronal cells.

INTRODUCTION

We have recently reported that acinic cell carcinoma of the human parotid gland contains argyrophil cells and expresses vasoactive intestinal polypeptide which functions as a neurotransmitter substance as well as a gastrointestinal hormone (1–3). In addition, we have found that a certain small cell undifferentiated carcinoma of the minor salivary gland contains exocrine, neuroendocrine, and squamous cells (4). These findings suggest that some of human salivary gland tumors may be of neuroectodermal origin, e.g., a neural crest cell.

In our laboratory, a neoplastic human salivary gland cell line, established from a human submandibular salivary gland, has been used as a model for studying mechanisms regulating cytodifferentiation in salivary glands (5–11). Thus, we have taken much interest in whether this cell line expresses neural cell phenotypes. In this communication, we report the expression of neurofilaments in a neoplastic human salivary intercalated duct cell line or its derivatives and the effect of NGF on cellular proliferation and phenotype.

MATERIALS AND METHODS

Culture and Media. HSG and its derivatives, HSG-AZA1 and HSG-AZA3, which were induced by treatment of HSG cells with 5-azacytidine, were grown in Eagle's minimal essential medium 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, HSG-AZA1, and HSG-AZA3 cells were described previously (5–12).

When the HSG cells had 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 (5, 8, 12). Of 10 colonies isolated, the one clone which showed the most stable growth was used for the current study. This HSC clone had an ultrastructure specific to the intercalated duct cells of human salivary gland; expressed biological markers such as carci noembryonic antigen, secretory component, and lactoferrin; and formed an adenocarcinoma upon transplantation of the cells into nude mice, as described previously in the parental HSG cells (5, 12).

The HSG cells were cultured in the continued presence of 5 μM 5-azacytidine at 37°C for 5 days and then the treated cells were subcultured once in the growth medium without 5-azacytidine. The HSG-AZA1 and HSG-AZA3 cells were cloned from the subculture using a dilution plating technique. The HSG-AZA1 cells exhibit a phenotype similar to that of myoepithelial cells such as microfibrils and myosin and form a myoepithelioma upon transplantation of the cells into nude mice, as described previously (10). The HSG-AZA3 cells have a phenotype specific to acinar cells such as the expression of amylase and transepithelial secretion of the cells into nude mice results in production of acinic cell carcinoma, as described elsewhere (10).

A human neuroblastoma cell line, IMR-32 (13), was grown in Eagle’s minimal essential medium containing 5% fetal bovine serum, 5% newborn calf serum, 2 mM L-glutamine and nonessential amino acids (Flow Laboratories, Rockville, MD) in a 5% CO2 incubator at 37°C. IMR-32 cells were purchased from Flow Laboratories.

Treatment of Cells with Nerve Growth Factor. NGF derived from mouse submandibular salivary gland (Wako Pure Chemical Co., Osaka, Japan) was used in the current study. This was dissolved in medium, maintained as a stock 100-μg/ml solution, and was added to culture medium to yield the required concentration.

Cells (105/dish) were seeded in 60-mm plastic Petri dishes (Falcon Labware, Oxnard, CA) at Day 0 in the growth medium. At Day 1, NGF was added at various concentrations (500–125 ng/ml). The cells were dissociated with trypsin solution on Day 10 and then were routinely subcultured in the growth medium with the continued presence of NGF in the initial concentration. During the entire experimental period, the growth medium containing each concentration of NGF at the initial treatment was changed every other day.

Plating Efficiency. To study the plating efficiency of cells on plastic surface, cells were dispersed into a single cell suspension with trypsin solution and were plated into a 60-mm plastic Petri dish (Falcon) at a density of 105 or 106 cells/ml of growth medium. They were incubated for 10 days at 37°C in a 5% CO2 incubator. After being stained with 10% (v/v) Giemsa solution, the colonies formed were grossly counted.

The plating efficiency of cells in semisolid agar medium was studied with the colony-forming technique. Briefly, suspensions of 105 or 106 cells in a growth medium containing 0.3% (w/v) Special Agar Noble (Difco Laboratories, Detroit, MI) were poured onto the agar medium, which had been hardened by the addition of 0.6% (w/v) agar into the growth medium. After about 20 days of incubation in a 5% CO2 incubator at 37°C, the colonies formed were counted under an inverted light microscope.
microscope. Colonies consisting of more than 10 cells were counted in duplicate dishes.

**Indirect Immunofluorescence Technique.** Cells grown in glass coverslides were fixed in acetone at −20°C for 10 min and then subjected to indirect immunofluorescence microscopy for detection of neurofilaments, tubulin α and β chain, HNK-1 (Leu-7) antigen, α and β chain of S-100 protein, or laminin in the cells. The fixed cells were incubated for 1 h at 37°C with the first antibodies, with a subsequent washing step between the incubations. The first antibodies used in this single label experiment were: mouse monoclonal IgG antibody to rat neurofilament, M, 200,000 (Enzo Biochem. Inc., New York, NY; diluted 1:20); mouse monoclonal IgG1 antibody to α or β chain of tubulin from native chick brain microtubules (Amersham International plc, Amersham, Buckinghamshire, England; diluted 1:20); mouse monoclonal IgM antibody to Leu-7 antigen from the HSB-2 T-lymphoblastoid cell line (Becton-Dickinson, Inc., Sunnyvale, CA; diluted 1:20); mouse monoclonal IgG1 antibody to α or β chain of S-100 protein (Ohtsuka Assay Laboratories, Inc., Tokushima, Japan; diluted 1:5); or rabbit antiserum to laminin (E-Y Laboratories, Inc., San Mateo, CA; diluted 1:100). These preparations were then incubated for 1 h at 37°C with the second antibodies, again with a subsequent washing step. The labeled second antibodies were: fluorescein-conjugated goat anti-mouse IgG and IgM (Cappel Laboratories, Cochranville, PA; diluted 1:25); or fluorescein-conjugated goat anti-rabbit IgG (Dakopatts, Copenhagen,
Fig. 2. Indirect immunofluorescence microscopy of HSG, HSG-AZA1, HSG-AZA3, and IMR-32 cells. Single label immunofluorographs showing the expression of neurofilaments (a–d), tubulin α chain (c–h), and tubulin β-chain (i–l) in the cells. a, e, i, HSG; b, f, j, HSG-AZA1; c, g, k, HSG-AZA3; d, h, l, IMR-32. First antibodies used: a–d, mouse monoclonal anti-neurofilaments; c–h, mouse monoclonal anti-α chain of tubulin; i–l, mouse monoclonal anti-β chain of tubulin. × 640.

Denmark; diluted 1:25). After a second series of washes, the samples were mounted with 50% glycerol in PBS. According to the specification sheet from the manufacturer, the mouse monoclonal IgG antibody to M, 200,000 rat neurofilament recognizes an epitope common to a triplet of proteins (M, 68,000, 160,000, and 200,000) composing neurofilaments.

The double-antibody labeling technique was performed essentially as described by Ramaekers et al. (14). The fixed cells were incubated with
Fig. 3. Indirect immunofluorescence microscopy of HSG, HSG-AZA1, HSG-AZA3, and IMR-32 cells. Single label immunofluorographs showing the expression of \( \alpha \) chain of S-100 protein (a–d), \( \beta \) chain of S-100 protein (e–h), HNK-1 antigen (i–l) and laminin (m–p) in the cells. a, e, i, m, HSG; b, f, j, n, HSG-AZA1; c, g, k, HSG-AZA3; d, h, l, p, IMR-32. First antibodies used: a–d, mouse monoclonal anti-\( \alpha \) chain of S-100 protein; e–h, mouse monoclonal anti-\( \beta \) chain of S-100 protein; i–l, mouse monoclonal anti-HNK-1; m–p, rabbit anti-laminin. × 440.
Arrows (A), positions corresponding to neurofilament triplets. Small arrow (B), weight in thousands. C), position corresponding to tubulin α chain (B) or β chain (C). A, molecular weight in thousands.

After an incubation step of 30 min with mouse monoclonal antibody to rat neurofilament (Enzo; diluted 1:20); rabbit antiserum to CEA (Dakopatts; diluted 1:100); rabbit antiserum to amylase from human saliva (Nordic Immunology, Tilburg, The Netherlands; diluted 1:200); or rabbit antiserum to bovine uterus myosin (Paesel GmbH & Co., Frankfurt, West Germany; diluted 1:50).

After an incubation step of 30 min with mouse monoclonal anti-neurofilament and rabbit antiserum to CEA, mouse monoclonal anti-neurofilament and rabbit antiserum to myosin, or mouse monoclonal anti-neurofilament and rabbit antiserum to amylase in a humidified box at a room temperature, the samples were washed with PBS containing 0.25% Triton X-100 in 2 subsequent washing steps of 10 min each and thereafter were washed with the PBS alone. These preparations were then incubated for another 30 min with the second antibodies, and then the first antibodies, with a subsequent washing step between the incubations. The labeled second antibodies (Cappel) were: fluorescein-conjugated goat anti-mouse IgG and IgM (diluted 1:25 each); and rhodamine-conjugated goat anti-rabbit IgG (diluted 1:20). After a second series of washes, the samples were mounted with 50% glycerol in PBS. Cells were examined under a Nikon microscope, equipped with epifluorescent illumination (HBO 100-W bulb), by using the appropriate filter systems for fluorescein and rhodamine fluorescein. Overlap between the 2 channels was checked by using cells labeled with a single antibody. Tri-X film from Kodak was used for photographs.

To test the specificities of monoclonal antibodies used for the detection of neurofilaments, α and β chain of tubulin, HNK-1 antigen, and α and β chain of S-100 protein, specific antibodies were replaced by either PBS, unrelated mouse monoclonal IgG1 antibody to herpes simplex virus type 1 (Cappel), or unrelated mouse monoclonal IgM antibody to hepatitis B surface antigen (Bioclone Australia Pty., Ltd., Marrickville, Australia). In addition, mouse monoclonal antibodies to neurofilament and tubulin α or β chain were absorbed with IFs samples prepared from IMR-32 cells as described below. Mouse monoclonal antibody to α or β chain or S-100 protein was absorbed with S-100 protein from bovine brain (Calbiochem-Behring, Inc., La Jolla, CA).

Mouse monoclonal antibody to HNK-1 antigen was absorbed with 107 large granular lymphocytes isolated from human peripheral blood lymphocytes according to the method of Kumagai et al. (15) using Percoll discontinuous density gradient centrifugation. The rabbit antiserum used for the detection of amylase, myosin, and laminin were absorbed with each corresponding antigen. Antibody solution diluted 5-fold in PBS (1 ml) was mixed with 200 μg of specific antigen and incubated at room temperature for 2 h. Thereafter, they were centrifuged at 500 × g for 15 min at 4°C and then filtered through a Millipore HA type membrane (Millipore Co., San Francisco, CA) to remove microaggregates. The supernatants were used as absorbed antisera for the current study. These antibody samples gave negative results. Amylase from human saliva and myosin from bovine uterus muscle were purchased from Sigma Chemical Co., Saint Louis, MO, and laminin from Engelbreth-Holm-Swarm murine tumor was purchased from Collaborative Research, Inc., Lexington, MA. The rabbit antiserum to CEA was absorbed with normal human spleen extract to remove nonspecific cross-reacting antigen as described previously (6). A blocking test in the use of anti-CEA serum was provided by incubating parallel samples with the anti-CEA serum absorbed with a CEA extract of human colon cancer tissues. The negative controls were provided by incubations of parallel samples with a relevant dilution of normal rabbit serum instead of the rabbit specific antiserum of the initial incubation.

**Morphological Observation.** The monolayer cell culture was fixed in methanol and stained with 0.1 m phosphate-buffered Giemsa (pH 6.8) or with silver according to Bodian's silver method (16, 17).

For transmission electron microscope, the cultured cells were prepared according to standard procedures. The finished preparations were observed under a Hitachi Model H-500 electron microscope.

For immunoelectron microscopy, the cell monolayers were washed in PBS, fixed with periodate-lysine-parafomaldehyde as described by McLean and Nakane (18) for 2 h at 4°C, and then washed for 1 h in 5 changes of PBS containing 10% (w/v) sucrose. Thereafter, cells were incubated overnight with mouse monoclonal antibody to rat neurofilaments (Enzo) at 4°C. After being washed in PBS for 30 min, cells were reacted with a horseradish peroxidase-labeled Fab' fragment of anti-mouse IgG (Cappel) 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% dimethylaminoazobenzene (Wako) 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. Ultrathin sections were examined without staining under a Hitachi electron microscope. A control experiment was done with normal rabbit serum.

**Immunoblotting.** The preparations enriched in IFs from cultured cells were performed according to the method of Franke et al. (19). The
procedure for this IFs preparation was described in detail previously (6). The presence of neurofilaments and tubulin \( \alpha \) or \( \beta \) chain in the IFs preparation was investigated by immunoblotting.

The procedure for sodium dodecyl sulfate:polyacrylamide gel electrophoresis used in the current study essentially followed the method of Laemmli (20). Proteins from gels were transferred to nitrocellulose paper as reported by Towbin et al. (21). Detection of neurofilaments and tubulin \( \alpha \) or \( \beta \) chain on strips was performed by an ABC method (22) using a mouse monoclonal antibody to \( M, 200,000 \) rat neurofilament and a mouse monoclonal antibody to tubulin \( \alpha \) or \( \beta \) chain from chick brain and Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to the procedures indicated by the manufacturer.

**RESULTS**

Neurofilaments and Biological Markers in Neoplastic Human Salivary Intercalated Duct Cell Line HSG and Its Derivatives Proliferating *in Vitro*. HSG cells and their derivatives, HSG-AZA1 and HSG-AZA3, as well as human neuroblastoma cell line IMR-32, which were cultured in a 5% CO\(_2\) incubator for 3 days at 37°C, were stained by Bodian’s silver method and examined by the immunofluorescent labeling for the expression of neurofilaments and biological markers such as tubulin, S-100 protein, HNK-1 antigen, or laminin. Figs. 1–3 show representative samples of the silver stain and immunofluorescent
appearance of HSG, HSG-AZAI, HSG-AZA3, and IMR-32 cells. All the examined cells stained with Bodian impregnation and revealed the presence of neurofilaments, tubulin α and β chain, and HNK-1 antigen in the cytoplasm and the expression of laminin along the cytoplasmic membrane. Although the presence of α and β chain of S-100 protein was not detected in HSG and HSG-AZA3 cells, HSG-AZAI cells expressed only the β chain of S-100 protein as described elsewhere (8). On the other hand, IMR-32 cells were found to contain only α chain of S-100 protein, but not β chain of S-100 protein, as observed previously in the brain and peripheral neurons (23).

Moreover, to confirm that the neurofilament and tubulin polypeptides which are reactive to antibody directed against M, 200,000 rat neurofilament, or tubulin α or β chain, exist in the cells these antibodies were applied to nitrocellulose transfers of sodium dodecyl sulfate-polyacrylamide gel electrophoresis containing the preparations enriched in IFs from HSG, HSG-AZAI, HSG-AZA3, or IMR-32 cells. After reaction of the nitrocellulose sheets with mouse monoclonal antibody to M, 200,000 neurofilament, followed by biotin-labeled anti-mouse IgG goat serum and ABC complex, a distinct reaction was seen with M, 68,000, 160,000, and 200,000 polypeptides of HSG, HSG-AZAI, HSG-AZA3, or IMR-32 cells (Fig. 4A, Lanes a–d). The respective monoclonal antibody to tubulin α or β chain bound to M, 57,000 and 54,000 polypeptides present in all the examined cells as reported elsewhere (24) (Fig. 4, B and C, Lanes a–d).

Since we have found that the expression of CEA in HSG cells, of myosin in HSG-AZAI cells, or of amylase in HSG-AZA3 cells can be detected by immunoperoxidase staining (5, 10), these cells as well as IMR-32 cells were further examined by double immunofluorescence labeling study, using a rabbit antiserum against CEA, myosin, or amylase and a mouse monoclonal antibody directed against M, 200,000 neurofilament. Fig. 5 shows some typical examples of HSG, HSG-AZAI, HSG-AZA3, or IMR-32 cells stained by the double label technique, illustrating the coexistence of neurofilaments and CEA in HSG cells, neurofilaments and myosin in HSG-AZAI and IMR-32 cells, and neurofilaments and amylase in HSG-AZA3 cells. The presence of CEA or amylase in IMR-32 cells could not be detected (data not shown).

Effect of NGF on Cellular Proliferation and Phenotype. When cultured in the growth medium with NGF, HSG-AZAI cells showed major morphological alteration. Fig. 6 shows representative samples with the morphological appearance of HSG-AZAI cells treated with various concentrations of NGF. Forty-eight h after the treatment, cells with long cytoplasmic processes began to appear in the cultured HSG-AZAI cells in the presence of 500 or 250 ng/ml of mouse NGF. On Day 10, the treated cells were passaged with trypsinization and were further cultured in the continued presence of NGF in the initial concentration. At Day 24, almost 100% of the cells cultured in the growth medium containing NGF formed markedly long cytoplasmic processes and grew in a mesh-like arrangement. Al-
though IMR-32 cells cultured in the presence of NGF showed a similar morphological alteration, treatment of HSG or HSG-AZA3 cells with NGF did not result in morphological alteration of the cells under the current experimental conditions.

Fig. 7 shows the growth curves of HSG-AZA1 cells in the presence and absence of NGF. HSG-AZA1 cells were cultured for 10 days in the continued presence of NGF (500 or 250 ng/ml) at 37°C. Then the treated cells were subcultured with trypsinization and were further cultured in the growth medium containing each concentration of NGF at the initial treatments or in the growth medium without NGF. A reduction in the growth rate of HSG-AZA1 cells cultured in the presence of NGF (500 or 250 ng/ml) was observed, especially after the initial cultivation for 10 days in the presence of NGF. After the removal of NGF from HSG-AZA1 cells cultured for 10 days in the growth medium with NGF, the normal growth rate was not restored within the current experimental period. The doubling times for HSG-AZA1 cells treated with 250 or 500 ng/ml of NGF for the initial 10 days were 45.7 and 47.8 h, respectively, while the doubling times for the untreated HSG-AZA1 cells were 43.9 h. The cultured HSG-AZA1 cells in the presence of NGF at a final concentration of 250 or 500 ng/ml formed colonies in semisolid agar with efficiencies of 13.5 and 4.9%, respectively, while the colony-forming ability of the untreated HSG-AZA1 cells in semisolid agar was 17.1% (Table 1). The percentages of colony formation on a plastic surface of HSG-AZA1 cells cultured in the continued presence of 250 or 500 ng/ml of NGF were 53.5 and 23.3%, respectively, while the untreated HSG-AZA1 cells formed colonies on a plastic surface at 37°C, the colonies formed were counted.

The apparently dispersed cells (10³ or 10⁴) were plated into a 60-mm plastic Petri dish and incubated at 37°C in a 5% CO₂ incubator. Twenty-four h after plating, 0.5 ml of 0.3% (w/v) agar medium containing NGF in the initial concentration was added to the cultures. After 20 days of incubation at 37°C, the colonies formed were stained with Giemsa solution and counted.

The duplicate sets of numbers in Columns 4 and 5 indicate results of 2 different experiments.

### Table 1 Colony-forming ability of HSG, HSG-AZA1, HSG-AZA3, and IMR-32 cells in the presence of NGF

<table>
<thead>
<tr>
<th>Cells</th>
<th>NGF concentration (ng/ml)</th>
<th>No. of colonies in semisolid agar</th>
<th>No. of colonies on plastic surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSG-AZA1</td>
<td>500 1 × 10⁴</td>
<td>4, 3</td>
<td>20, 18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48, 50 (4.9%)</td>
<td>240, 225 (23.3%)</td>
</tr>
<tr>
<td></td>
<td>250 1 × 10⁴</td>
<td>8, 14</td>
<td>50, 53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>128, 143 (13.5)</td>
<td>540, 629 (58.5)</td>
</tr>
<tr>
<td></td>
<td>0 1 × 10⁴</td>
<td>20, 15</td>
<td>74, 69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>164, 178 (17.1)</td>
<td>784, 741 (76.3)</td>
</tr>
<tr>
<td>IMR-32</td>
<td>500 1 × 10⁴</td>
<td>8, 7</td>
<td>10, 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>89, 88 (8.9%)</td>
<td>140, 131 (13.6)</td>
</tr>
<tr>
<td></td>
<td>250 1 × 10⁴</td>
<td>10, 12</td>
<td>25, 33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>112, 126 (11.9)</td>
<td>363, 318 (34.1)</td>
</tr>
<tr>
<td></td>
<td>0 1 × 10⁴</td>
<td>9, 10</td>
<td>40, 33</td>
</tr>
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<td></td>
<td></td>
<td>128, 140 (13.4)</td>
<td>490, 410 (45.0)</td>
</tr>
<tr>
<td>HSG-AZA3</td>
<td>500 1 × 10⁴</td>
<td>11, 10</td>
<td>73, 85</td>
</tr>
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<td></td>
<td>140, 156 (14.8)</td>
<td>698, 734 (71.6)</td>
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<tr>
<td></td>
<td>250 1 × 10⁴</td>
<td>18, 14</td>
<td>78, 81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>158, 178 (16.8)</td>
<td>720, 807 (76.4)</td>
</tr>
<tr>
<td></td>
<td>0 1 × 10⁴</td>
<td>23, 20</td>
<td>75, 68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>266, 216 (24.1)</td>
<td>851, 821 (83.6)</td>
</tr>
<tr>
<td>HSG</td>
<td>500 1 × 10⁴</td>
<td>26, 15</td>
<td>61, 56</td>
</tr>
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<td></td>
<td>174, 208 (19.1)</td>
<td>736, 570 (65.3)</td>
</tr>
<tr>
<td></td>
<td>250 1 × 10⁴</td>
<td>16, 22</td>
<td>80, 63</td>
</tr>
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<td></td>
<td>218, 205 (21.2)</td>
<td>880, 812 (84.6)</td>
</tr>
<tr>
<td></td>
<td>0 1 × 10⁴</td>
<td>28, 24</td>
<td>90, 91</td>
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<tr>
<td></td>
<td></td>
<td>258, 253 (25.6)</td>
<td>914, 883 (89.9)</td>
</tr>
</tbody>
</table>

*Cells were mixed with 1 ml of 0.3% (w/v) agar medium containing NGF (500 or 250 ng/ml) and then overlaid onto a basal layer that had been made by pouring 2 ml of 0.6% (w/v) agar medium into a 35-mm plastic Petri dish. Ten days after plating, 0.5 ml of 0.3% (w/v) agar medium containing NGF in the initial concentration was added to the cultures. After 20 days of incubation at 37°C, the colonies formed were counted.

* The singly dispersed cells (10⁴ or 10⁵) were plated into a 60-mm plastic Petri dish and incubated at 37°C in a 5% CO₂ incubator. Twenty-four h after plating, NGF (500 or 250 ng/ml) was added. At Days 4, 7, and 10, the medium change was performed with the growth medium containing NGF in the initial concentration. After 14 days of incubation at 37°C, visible colonies formed were stained with Giemsa solution and counted.

* Numbers in parentheses, percentages of efficiency of colony formation of the cells in semisolid agar or on plastic surface.

* The duplicate sets of numbers in Columns 4 and 5 indicate results of 2 different experiments.

- Cells in the continued presence of NGF (500 or 250 ng/ml) were stained with Giemsa solution and counted.
- The duplicate sets of numbers in Columns 4 and 5 indicate results of 2 different experiments.
Fig. 8. Ultrastructural investigation of HSG-AZA1 and IMR-32 cells treated with NGF. 

**a, b**, HSG-AZA1 cell treated with 500 ng/ml NGF for 20 days. Cell with numerous microfibrils in addition to microtubule bundles and long cytoplasmic processes. × 4,800. c, high power view of b. × 9,500. 

**d**, untreated HSG-AZA1 cells × 4,800. e, f, immunoelectron micrographs without staining by uranyl acetate and lead hydroxide, demonstrating the presence of microfilamentary systems reactive to mouse monoclonal anti-neurofilament (e) and microtubule bundles reactive to mouse monoclonal anti-α chain of tubulin (f) in the HSG-AZA1 cells treated with 500 ng/ml NGF for 20 days, e, f. × 36,000. 

**g**, IMR-32 cells treated with 500 ng/ml NGF for 20 days. Neurite with numerous microfilaments and microtubule bundles (large arrow) and synaptic-like structure (small arrows) between neighboring cells. × 4,800. h, high power view of g. × 16,000.
tron microscopic investigation, the expression of microfilamentary systems reactive to mouse monoclonal anti-neurofilament (Fig. 8e) and microtubules reactive to mouse monoclonal anti-α chain or anti-β chain of tubulin (Fig. 8f) was found to be present in the HSG-AZA1 cells. On the other hand, the ultrastructure of the IMR-32 cells treated with 500 ng/ml NGF for 20 days represented typical neuronal cells with formation of neurites containing ample neurofilament and microtubule bundles or with formation of synaptic-like structures between neighboring cells (Fig. 8, g and h).

DISCUSSION

This paper reports that the neoplastic human salivary intercalated duct cell line HSG as well as its derivatives HSG-AZA1 and HSG-AZA3 are argyrophil cells and express some antigens specific to neuronal cells such as a triple of neurofilament polypeptides and tubulin α or β chain as well as HNK-1 antigen. Mammalian neurofilaments, which represent a class of antigenically and biochemically distinct intermediate-sized filaments, are found principally in neurons and are composed of three polypeptides with approximate molecular weights of 68,000, 160,000, and 200,000 (26). In addition, it has been suggested that M, 200,000 polypeptide probably has a more specialized role in neurofilament architecture and function than the other two triplet polypeptides on the basis of differential expression of neurofilament triplet polypeptides in brain development (27, 28). Moreover, it has been known that the binding site for the Bodian stain is located in the b domain in the extended tail segment, which is a unique feature of neurofilament polypeptides (26, 29). Furthermore, it has been reported that the assembly into microtubules of the tubulin polypeptides, being composed of α and β chains (24), is an essential step in the growth of neuronal processes (30–32).

It has been shown that HNK-1 antigen, which was initially determined as a differentiation antigen of human natural killer and killer cells (33), functions as one of the nervous system cell adhesion molecules (34, 35) and is involved in interactions between neural crest cells and laminin substrates (36). In addition, it has been reported that the monoclonal anti-HNK-1 reacts with myelinated nerves, other normal tissues, tumors of neuroectodermal origin, and the amine precursor uptake and decarboxylation system (37, 38). Moreover, it has been demonstrated that normal benign and malignant prostate tissues as well as small cell lung carcinoma express HNK-1 antigen (39, 40). The presence in the HSG-AZA1 cells of the β chain of S-100 protein, which is usually found in glial and not neuronal cells (23), is somewhat inconsistent with a neuronal phenotype. However, it can be considered that HSG-AZA1 cells show the coexpression of the biological markers of neuronal and salivary myoepithelial cells.

It has been described that besides the peripheral nervous system, the neural crest cells give rise to a wide variety of structures, e.g., all the melanocytes of the body except those of pigmented retina and of the central nervous system, certain endocrine and paracrine cells such as calcitonin-producing cells, type 1 cells of the carotid body or adrenal medulla cells; in addition, brain meninges, facial and visceral arch skeleton and dermis, musculoconnective wall of the large arterial trunks arising from the aortic arches, and the connective tissues of the buccal and pharyngeal region, including that of the salivary, thyroid, parathyroid, and thymus gland (41, 42). Thus, the above findings strongly suggest that HSG cells and their derivatives examined in the current study may be derived from the neural crest cell, although it cannot be neglected that the presence of neurofilaments in the cells may be caused by abnormalities in regulating gene expression associated with IFs rather than the cellular origin. It has been reported that all of the IFs proteins such as keratin, desmin, vimentin, glial fibrillary acidic protein, and neurofilament have a conserved M, 40,000 rod-shaped domain, which has probably been derived from the ancestral IFs gene (26, 43–46).

In the current study, it has been shown that NGF treatment of HSG-AZA1 cells with a myoepithelial cell phenotype results in conversion into the cells with long cytoplasmic processes similar to neurites as observed in the PC12 rat pheochromocytoma cells or human neuroblastoma cells, which were treated with NGF (47–49). It has been reported that by 1 week of exposure of NGF (50 ng/ml), PC12 cells cease to multiply and begin to extend neuronal-like processes, and over the next 2 weeks of treatment, the number, length, and density of such processes continue to increase until at least 80% of the cells have responded (47). On the other hand, it has been demonstrated in IMR-32 cells that 100 ng/ml of NGF is the lower threshold for determining significantly a measurable degree of morphological differentiation and that NGF at 2000 ng/ml yields an optimum response, in which IMR-32 cells require 8–10 days for the expression of maximal differentiation (49). Based on the above findings reported previously by other investigators, it can be considered that a response to NGF in morphological differentiation is more vigorous in PC12 cells than in IMR-32 or HSG-AZA1 cells, although the reason remains to be proved in the current study. Moreover, we have found a reduction in anchorage-independent and anchorage-dependent growth potentials of the treated HSG-AZA1 cells, as compared with those of the untreated control. It has been reported that NGF is essential for the survival and differentiation of a number of neural crest derivatives, including sympathetic and sensory neurons (50, 51). Moreover, it has been demonstrated in the study with clonal PC12 rat pheochromocytoma cells and their variants that NGF possesses both antimitogenic and mitogenic activities (52). On the other hand, the phenotype of HSG and HSG-AZA3 cells were hardly affected with NGF treatment, although a substantial affection for the anchorage-independent and anchorage-dependent growths of the NGF-treated cells was observed.

The HSG-AZA1 and HSG-AZA3 cells used in the current study were prepared from the parental HSG cells by treatment with 5-azacytidine. In general, it is well known that the treatment of mammalian cells with 5-azacytidine results in hypomethylation of DNA, which causes expression of silent genes of cells (53–56). Since it has been shown that total genomic DNA methylation levels in HSG-AZA1 and HSG-AZA3 cells are significantly decreased when compared with the level of the parental HSG cells (10), it can be considered that the genes involved in the induction of morphological alteration through NGF signal transduction may be expressed in HSG-AZA1 cells as a consequence of DNA hypomethylation. Thus, we focused on the effect of NGF on the phenotype of HSG-AZA1 cells in comparison to that of NGF-treated IMR-32 cells. While the neurites formed in NGF-treated IMR-32 cells were densely packed with neurofilaments as well as microtubules which are reported to be required for the growth of neurites (30, 32), NGF-treated HSG-AZA1 cells formed long cytoplasmic processes with ample microfilbrils, which had numerous microvilli. In addition, the presence of laminin in the HSG-AZA1 and IMR-32 cells was clearly observed, although HSG and HSG-AZA3 cells also expressed laminin. It has been shown that

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laminin promotes neuronal process outgrowth (57–61). To the best of our knowledge, this is the first report indicating the expression of neurofilaments and positive staining with Bodian impregnation in human salivary cell lines proliferating in vitro. Especially, the current study demonstrates that among the various types of human salivary cells examined, only HSG-AZA1 cells with a myoepithelial cell phenotype respond to NGF and form the long cytoplasmic processes. Because of the unique characteristics of HSG cells and their derivatives, these cells may contribute to an improvement in our understanding of the mechanisms associated with cytodifferentiation or tumor formation in human salivary glands and of the mechanisms regulating the functions of human salivary myoepithelial cells.

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Expression of Neurofilaments in a Neoplastic Human Salivary Intercalated Duct Cell Line or Its Derivatives and Effect of Nerve Growth Factor on the Cellular Proliferation and Phenotype

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