Intermediate-sized Filaments and Specific Markers in a Human Salivary Gland Adenocarcinoma Cell Line and Its Nude Mouse Tumors

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ABSTRACT

The adenocarcinoma cell line HSG from human salivary gland, which proliferates in vitro or in nude mice, was examined by the immunoperoxidase method for the expression of three different types of intermediate-sized filaments (IFs) and of specific antigens such as carcinoembryonic antigen, S-100 protein, secretory component, lactoferrin, myosin, tropomyosin, and actin. The cultured HSG cells were found to express three different types of IFs defined by antibodies to keratin, vimentin, and desmin. In HSG cells proliferating in vitro at 34°C and 37°C but not at 39°C, the expression of tropomyosin and carcinoembryonic antigen was observed, although myosin and S-100 protein were not detected. The expressions of actin, lactoferrin, and secretory component were restricted to cultured HSG cells at 39°C and 37°C, respectively. Transplantation of HSG cells into nude mice resulted in the establishment of a nude mouse system with malignant characteristics such as invasion and metastasis. The expression of IFs in the primary tumors was restricted to keratin and desmin IFs, whereas coexpression of keratin, vimentin, and desmin IFs was observed in some neoplastic cells present in the metastatic tumors in regional lymph nodes and lung. In addition, expression of actin, myosin, tropomyosin, and S-100 protein was found in the metastatic tumors, whereas myosin and S-100 protein were not detected in the primary tumors. Moreover, the metastatic tumors were almost occupied by the neoplastic cells with oncocytic changes, although oncocytic change was not found in the cultured HSG cells and their primary tumors.

INTRODUCTION

It is well known that the epithelial tumors arising in human salivary glands show a variety of histopathological features and that their neoplastic cells are mostly composed of an admixture of transformed epithelial duct and myoepithelial cells. Since the cells that are pluripotent in differentiation such as the intercalated duct and myoepithelial cells are present in the salivary glands and can differentiate into the other types of cells such as squamous and chondroid cells (10, 15, 21, 28, 37), tumor formation in human salivary glands can be regarded as a consequence of neoplastic transformation and its proliferation in the process of differentiation of one or both of the 2 types of cells.

However, on the basis of their morphological findings in the surgical specimens of salivary gland neoplasms, many investigators have suggested that the epithelial tumors of salivary gland arise from the intercalated ducts and that the myoepithelial cells including duct-like epithelial cells function as supporters of the formation of certain salivary gland neoplasms (9, 11, 20, 22). This may indicate that the salivary gland neoplasms stem from the intercalated duct cells and that the neoplastic cells can represent both phenotypes of epithelial and mesenchymal cells. Recently, neoplastic epithelial duct cells of human salivary gland origin have been isolated and characterized (38). This cell line, HSG, has an ultrastructure similar to that of intercalated duct cells, and transplantation of HSG cells into the backs of athymic BALB/c nude mice results in a formation of tumors which are histopathologically interpreted as adenocarcinoma. Thus, by utilizing this cell line, we have conducted investigations of differentiation of salivary gland duct cells. In particular, we have taken much interest in the mode of expression of IFs and specific markers in this HSG cell line.

Recent studies on IFs have revealed that epithelial cells are characterized by keratin IFs, whereas mesenchymal cells contain mainly IFs of the vimentin type and IF constituents of muscle cells are the desmin type (3, 4, 13, 27, 36). Moreover, several investigators have recently reported coexpression of keratin and vimentin-type IFs in human metastatic carcinoma cells or in mouse keratinocytes in culture (12, 32). On the other hand, the neoplastic cells present in various types of salivary gland neoplasms have recently been found to express specific markers such as CEA and S-100 protein (5, 6, 8, 18, 25, 29, 30, 33). We have also detected CEA, SC, and LF in the HSG cells (35).

In the present communication, we report the coexpression of 3 different types of IFs and temperature-dependent expression of several specific markers in neoplastic intercalated duct cells (HSG) proliferating in vitro. In addition, we establish the nude mouse system with biological characteristics such as invasion and metastasis similar to those of human salivary gland adenocarcinoma, and we describe its expression and its characterization of IFs and specific markers in the nude mice tumors.

MATERIALS AND METHODS

Culture and Media. A neoplastic epithelial duct cell line, HSG, established from a human salivary gland was grown in Eagle's minimal essential medium supplemented with 10% calf serum and 2 mM L-glutamine in the presence of 5% CO2 in a temperature gradient incubator at various temperatures (34°C, 37°C, and 39°C). This incubator (Toyo Ind., Co., Tokyo, Japan) is composed of 6 chambers, and the tempera-
ture in each chamber is controlled gradiently. The temperature regulation is within the limits of ±0.2°C (SD). The temperature conditions, regulated by a thermister PID (proportional-plus-integral-plus-derivative-control) thermocontroller, are monitored by the automatic recording system throughout the experiments. The HSG cell population which were cultured in this incubator for 3 days at 34°C, 37°C, and 39°C had >90% viability as judged by trypsin blue exclusion. The HSG cells (38) were isolated from the submandibular salivary gland which was taken by surgical operation from a patient who had received therapeutic irradiation for carcinoma of the floor of the mouth. Transplantation of these cells into the backs of BALB/c nude mice results in the production of adenocarcinoma with a solid and trabecular pattern. This cell line HSG has been maintained in monolayer culture, and subculture has been made on 5- or 6-day intervals.

When these HSG cells accomplished 400 cell generations from the start of the culture, the colony formation of HSG cells in semisolid agar was performed, as described previously (38). Briefly, suspension of 10^5 or 10^6 cells in the growth medium containing 0.3% Special Agar Noble (Difco Laboratories, Detroit, MI) were poured into the agar medium which had been hardened by the addition of 0.6% agar into the growth medium. The colonies 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 30-mm plastic Petri dishes until confluent cell monolayers were formed. The growth medium was changed twice a week during that period. Of 10 colonies isolated, one clone which showed most stable growth was used for this study.

**Heterotransplantation of HSG Cells Into Nude Mice.** For the purpose of the present study, 3-week-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 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 the time ranging from 3 to 10 months after the inoculation of HSG cells. Tumors, regional and distant lymph nodes, and representative sections from various organs were fixed in phosphate-buffered 10% formalin.

**Immunoperoxidase Staining.** The IFs and specific markers of tumor cells were essentially investigated by the PAP method described by Sternberger et al. (39). The specific antigens chosen were keratin, vimentin, desmin, actin, myosin, tropomyosin, CEA, S-100 protein, LF, and SC.

The cultured cells were washed 3 times with PBS and fixed with 95% ethanol:aceton (4:6) for 10 min at 4°C. The paraffin-embedded specimens were cut in serial sections approximately 4 μm thick. The paraffin sections were deparaffinized with xylene and rehydrated step by step with descending concentrations of ethanol.

These preparations 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 antisera to each of the above-described antigens for 30 min at room temperature followed by a rinse with PBS for 30 min. Thereafter, goat anti-rabbit 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 (Medical Laboratories, Inc., Elkhart, IN), 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 10 ml Tris-HCl buffer (pH 7.6) for 5 min; after being washed with distilled water, these samples were counterstained with hematoxylin.

The following controls were used. The rabbit antisera to CEA was absorbed with normal human spleen extract to exclude nonspecific cross-reacting antigen as follows. Antibody sample (0.2 ml) was mixed with 100 μg of acetone powder prepared from normal human spleen and was incubated at room temperature for 2 h. Thereafter, this mixture was added with 0.8 ml of PBS, and the supernatant obtained by centrifuging at 500 x g for 15 min at 4°C was filtered through a type HA Millipore membrane (Millipore Co., San Francisco, CA) and used as an absorbed antisera for the present study. The acetone spleen powder was made as follows. Washed, diced spleen was ground with acetone in a mortar and filtered or squeezed onto coarse grade filter paper. After the deposit was washed 5 times with acetone, this was dried overnight at room temperature and used as the acetone spleen powder in 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 a CEA extract of human colon cancer tissues. The negative control was provided by incubations of parallel samples with a relevant dilution of normal rabbit serum instead of the rabbit specific antisera of the initial incubation. To test the specificities of antisera used for detection of actin, myosin, tropomyosin, S-100 protein, and LF, each specific antisera was replaced by either PBS, normal rabbit serum, or the specific antisera absorbed with each corresponding antigen. Antibody solutions diluted 5-fold in PBS (1 ml) were mixed with 200 μg of specific antigen and incubated at room temperature for 1 h. Thereafter, they were centrifuged at 500 x g for 15 min at 4°C and then filtered through a type HA Millipore membrane to remove microaggregates. The supernatants were used as absorbed antisera for the present study. These antibody samples gave negative results. Actin and myosin from chicken muscle, tropomyosin from rabbit muscle, and LF from human milk were purchased from Sigma Chemical Co., Saint Louis, MO. S-100 protein was purchased from Calbiochem-Behring, La Jolla, CA. For controls in staining with anti-SC serum, the primary antisera was replaced with normal rabbit serum. Also, parallel samples were treated with anti-human IgA, IgG, and IgM (Dakopatts, Copenhagen, Denmark) to rule out any positive reaction due to these substances.

Rabbit antisera to CEA, SC, and LF were purchased from Dakopatts, and the rabbit antisera to S-100 protein was purchased from Wako Pure Chemicals Co., Osaka, Japan. Rabbit antisera to human keratin, human vimentin, chicken desmin, chicken actin, chicken myosin, and chicken tropomyosin were purchased from Transformation Research Inc., Framingham, MA. In the experiments, anti-CEA and anti-S-100 protein sera were used at a dilution of 1:1000, anti-SC and anti-LF sera were used at a dilution of 1:500, anti-vimentin, anti-desmin, anti-actin, anti-myosin, and anti-tropomyosin sera were used at a dilution of 1:100; and anti-keratin serum was used at a dilution of 1:60.

**Light and Electron Microscopic Observation.** The paraffin sections of nude mice tumors were stained with hematoxylin and eosin.

For transmission electron microscopy, the tumor tissues were fixed in 4% glutaraldehyde buffered with 0.1 M phosphate (pH 7.3) containing 0.005 M calcium chloride for 1 h and then dissected into small pieces of about 2 cu mm. After these materials were washed in PBS, they were postfixed with 2% osmium tetroxide, dehydrated step by step with ethanol, and embedded in Epon 812. The ultrathin sections were cut on a LKB ultramicrotome and stained with uranyl acetate and lead hydroxide. The finished preparations were observed under a Hitachi Model H-500 electron microscope.

**Indirect Immunofluorescence Technique.** The double-antibody labeling technique was performed essentially as described by Ramaekers et al. (32). Cells grown in glass coverslides were fixed in acetone at −20°C for 10 min and then subjected to indirect immunofluorescence microscopy. The fixed cells were incubated with the first antibodies, with a subsequent washing step between the incubations. The first antibodies (Transformation Research) used in this double label experiment were: rabbit antisera to human vimentin and chicken desmin (diluted 1:20 each); a mouse monoclonal IgG antibody to human keratin (not diluted); or a mouse monoclonal IgM antibody to human vimentin (not diluted).

After an incubation step of 30 min with mouse monoclonal anti-keratin and rabbit antisera to human vimentin, mouse monoclonal anti-vimentin, and rabbit antisera to chicken desmin, or mouse monoclonal anti-keratin and rabbit antisera to chicken desmin in a humified box at

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**CANCER RESEARCH VOL. 45 AUGUST 1985**

3879
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, again with a subsequent washing step between the incubations. The labeled second antibodies (Cappel Laboratories, Cochranville, PA) 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-watt bulb), by using the appropriate filter systems for fluorescein and rhodamine fluorescence. Overlap between the 2 channels was checked by using cells labeled with a single antibody. Tri-X film from Kodak was used for photographs.

Immunoblotting. The preparations enriched in IFs were performed according to the method of Franke et al. (14). Briefly, HSG monolayers formed by culturing for 3 days at 37°C were rinsed twice in 10 mM Tris-HCl:140 mM NaCl buffer (pH 7.6) and then incubated for about 3 min in 10 mM Tris-HCl:140 mM NaCl buffer (pH 7.6) containing 1% Triton X-100 at room temperature. The cell residues remaining attached to the substrate were then incubated for 30 min at room temperature in 10 mM Tris-HCl:140 mM NaCl buffer (pH 7.6) containing 0.5% Triton X-100 and 1.5 mM KCl (pH 7.6). The pellet obtained by centrifuging the dispersed cell material at 3500 x g for 10 min was washed twice by brief dispersion in 10 mM Tris-HCl buffer (pH 7.6) followed by centrifugation. This material was further incubated for 15 to 25 min in 10 mM Tris-HCl buffer (pH 7.6) containing 0.2 mM MgCl2 and pancreatic DNase I (100 µg/ml; Worthington Biochemicals Corp., Freehold, NJ) at room temperature in order to digest the DNA present in these cytoskeletal ton-enriched preparations. For examination of the protein by SDS-PAGE, precipitates obtained with 5% cold trichloroacetic acid were washed with acetone and taken up in 10 mM Tris-HCl buffer (pH 7.6) with 2% SDS and 10 mM dithiothreitol. These preparations were sonicated and boiled for 2 min to solubilize IF proteins.

The procedure for SDS-PAGE used in the present study essentially followed the method of Laemmli (26). The slab gels measured 8.0 x 12.0 x 0.2 cm. The stacking gel contained 5% acrylamide (Wako Pure Chemicals Co.) and 0.13% N,N'-methylenebisacrylamide (Wako Pure Chemicals Co.). The separation gel contained 12.5% acrylamide and 0.1% N,N'-methylenebisacrylamide. Electrophoresis was performed at room temperature for 4 h at 4 mA/cm width of gel. The migration front was culated and boiled for 2 min to solubilize IF proteins.

RESULTS

IFs and Specific Markers in Human Salivary Gland Adenocarcinoma Cell Line HSG Proliferating In Vitro. HSG cells were cultured in a temperature gradient incubator for 3 days at 34°C, 37°C, or 39°C, and then they were examined by the PAP method for the expression of IFs and specific markers. Figs. 1 and 2 show representative samples of the immunohistochemical appearance of HSG cells which were cultured at various temperatures. Table 1 summarizes all of the results obtained by the immunohistochemical study on HSG cells.

As can be seen in Fig. 1, a to f, and Table 1, cultivation of HSG cells at 34°C or 37°C resulted in the coexpression of 3 different types of IFs defined by the antisera to keratin, vimentin, and desmin. Although only IFs of the desmin type disappeared in the cultured HSG cells at 39°C, both keratin and vimentin were detected in the cultivation of HSG cells at 39°C. Fig. 1, g to i, illustrates the mutual exclusiveness of the reactions of 3 antisera to keratin, vimentin, or desmin on frozen sections from normal human oral tissues. It is noted that keratin-specific antibody gives very strong positive staining for epithelium and that each antisera to vimentin or desmin reacts exclusively with submucosal mesenchymal tissue or with muscle tissues including smooth muscle cells present in the vascular region, respectively.

On the other hand, we have already demonstrated the presence of CEA, LF, and SC in the cultured HSG cells at 37°C (35). Thus, HSG cells were examined by the PAP method for expression and its temperature dependency with these specific markers including cytoskeletal proteins such as actin, myosin, and tropomyosin. As shown in Fig. 2 and Table 1, the expression of actin was clearly observed in the cultured HSG cells at 39°C, whereas actin was never detected in HSG cells which were cultured at either 34°C or 37°C. The expression of tropomyosin was demonstrated in the HSG cells which were cultured at either 34°C or 37°C, whereas tropomyosin did not occur in cultivation of HSG cells at 39°C. The expression of CEA was demonstrated in the cytoplasms of HSG cells which were cultured at either 34°C or 37°C but not at 39°C. The expression of LF and SC was restricted to the cultured HSG cells at 37°C. However, myosin and S-100 protein were not observed in HSG cells under any experimental conditions used for the present study.

Since it could be considered from the results obtained by the PAP method that cultured HSG cells express simultaneously 3 different types of IFs, cultured HSG cells at 37°C were further examined by double immunofluorescence labeling by using vimentin and desmin antibodies raised in a rabbit and a mouse monoclonal antibody directed against human keratin or human vimentin. Fig. 3 shows some typical examples of cultured HSG cells stained by the double label technique, illustrating the co-

<table>
<thead>
<tr>
<th>Specific antigens</th>
<th>34°C</th>
<th>37°C</th>
<th>39°C</th>
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<tbody>
<tr>
<td>Keratin</td>
<td>95</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>Vimentin</td>
<td>95</td>
<td>95</td>
<td>75</td>
</tr>
<tr>
<td>Desmin</td>
<td>90</td>
<td>95</td>
<td>0</td>
</tr>
<tr>
<td>Actin</td>
<td>0</td>
<td>0</td>
<td>75</td>
</tr>
<tr>
<td>Myosin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>95</td>
<td>95</td>
<td>0</td>
</tr>
<tr>
<td>CEA</td>
<td>80</td>
<td>95</td>
<td>0</td>
</tr>
<tr>
<td>S-100 protein</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LF</td>
<td>0</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>SC</td>
<td>0</td>
<td>2.5</td>
<td>0</td>
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The same was true for vimentin and desmin. It also was obvious that keratin, vimentin, and desmin are distributed differently throughout the cytoplasm of the cell. Surprisingly, many HSG cells gave the impression that 3 different IF systems co-localize. Throughout the cytoplasm of the cell. Surprisingly, many HSG cells gave the impression that 3 different IF systems co-localize.

Moreover, to confirm that intermediate-size filament proteins reactive to antibodies directed against keratin, vimentin, and desmin exist in the cultured HSG cells; these antisera were applied to nitrocellulose transfers of SDS-PAGE containing the preparation enriched in IFs from cultured HSG cells. After reaction of the nitrocellulose sheets with rabbit anti-keratin antibodies, followed by goat anti-rabbit IgG and PAP complex, a distinct reaction is seen with M, 46,000, 56,000 and 67,000 polypeptides of cultured HSG cells (Fig. 4, Lane a). Rabbit anti-vimentin antibodies bound to a M, 59,000 polypeptide present in cultured HSG cells, and rabbit anti-desmin antibodies bound to a M, 53,000 polypeptide (Fig. 4, Lanes b and c).

IFs and Specific Markers in the HSG Nude Mice Tumors. Inoculation of 10^7 HSG cells s.c. into nude mice resulted in production of lobulated tumor masses 8 to 13 mm in diameter in the inoculation site about 30 days after transplantation. These tumors became aggressively enlarged and, about 3 months after the inoculation, a majority of the tumor-bearing mice had formed ulcers in the outer skin adjacent to the tumor. However, these ulcers had a tendency to form a crest and were encapsulated by fibrous tissue. In addition, the formation of new tumor masses in the areas corresponding to axillary lymph nodes in the homolateral or less frequently bilateral was observed in almost all of the tumor-bearing mice. Since the tumor masses which could be considered the axillary lymph node metastasis became expansively enlarged, the animals were sequentially killed by cervical dislocation, and autopsy was performed.

The primary tumors which occurred in the sites inoculating HSG cells were histopathologically interpreted as adenocarcinoma with solid and trabecular pattern as described previously (38) (Fig. 5a). The tumor was composed of almost cuboidal cells with occasional anisocytism and mitosis. In addition, a group of larger cells which had pale-staining eosinophilic cytoplasm appeared in some areas of the tumor tissue. Although the tumor was surrounded by the pseudocapsule, some groups of neoplastic cells frequently invaded the pseudocapsule or occasionally infiltrated the surrounding fibrous and muscular tissues (Fig. 5b). Moreover, in all of 15 mice exhibiting macroinvasion into the outer skin, tumors invaded the local lymphatic channels which appeared dilated and filled with the clusters of neoplastic cells (Fig. 5c). Lymph node involvement was found in 13 of the examined 15 mice, which was restricted to the axillary lymph nodes. This was characterized by the presence of neoplastic cells in the peripheral sinus of lymph nodes with extension into the paraaortal and medullary areas. Nodal capsular invasion and tumor extension into the surrounding tissue was also observed. The metastatic tumors were histopathologically interpreted as adenocarcinoma with oncocytic change (Fig. 5d). The tumor was composed of sheets of predominantly polygonal or spindle-shaped large cells with occasional mitoses, the cytoplasm of which contained many eosinophilic granules. Ultrastructurally, numerous mitochondria with crescent-shaped cristae or concentric tubular cristae were observed in the cytoplasm of neoplastic cells (Fig. 5e). Two neighboring tumor cells were often attached with continuous tight junction, and distinct desmosomes were scant. Intercellular digitations formed by infoldings of cytoplasmic processes were sometimes observed. The nuclei were oval, and the nuclear chromatin was evenly distributed. In the lungs of 2 of the examined 15 mice, metastases were found in areas adjacent to small bronchi and bronchioli (Fig. 5f). A majority of the metastatic tumors present in the lung showed morphological characteristics identical to those in the lymph nodes.

The HSG cells used for this study were found to express actin, tropomyosin, LF, and SC, including 3 different types of IFs. Thus, a search for these antigens including myosin and S-100 protein on tissue sections of the primary and metastatic tumors was performed immunohistochemically. As shown in Fig. 6 and Table 2, keratin and desmin were observed in the neoplastic cells present in the primary tumors, although vimentin was not de-

Table 2

Summary of the immunohistochemical characteristics of the primary and metastatic tumors, which were produced by inoculation of HSG cells into nude mice.

<table>
<thead>
<tr>
<th>Specific antigens</th>
<th>Primary tumors</th>
<th>Lymph nodes</th>
<th>Lung</th>
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<tbody>
<tr>
<td>Keratin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vimentin</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Desmin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Actin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Myosin</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CEA</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S-100 protein</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LF</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SC</td>
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*+, positive; -, negative.
The tissue sections from primary tumors showed positive stainings of actin, tropomyosin, and CEA (Fig. 7). On the other hand, in addition to the cytoskeletal proteins such as actin, myosin, and tropomyosin, the expression of 3 different types of IFs including S-100 protein was detected in some neoplastic cells present in the metastatic tumors in the lymph nodes and lung, although CEA was not detected in the metastatic tumors (Figs. 6 and 7).

**DISCUSSION**

This paper reports expression and its characterization of IFs and specific markers in a neoplastic epithelial duct cell line (HSG) of human salivary gland in origin, which propagate in vitro and in nude mice. Since Hanna (17) has reported that tumors which are nonmetastatic in nude mice older than 10 weeks of age will metastasize at a high frequency if inoculated into 3-week-old animals, we attempted to establish a nude mouse system with biological actions similar to those of the adenocarcinoma arising from human salivary glands by inoculating HSG cells into the younger animals because transplantation of HSG cells into the backs of nude mice results in a formation of adenocarcinoma. Consequently, the nude mouse system established in the present study represented growth patterns such as local invasion and metastasis into the regional lymph nodes or lung.

The data presented here provide the evidence for coexpression of keratin, vimentin, and desmin in the HSG cells propagating in vitro. Although coexpression of keratin- and vimentin-type IFs has been demonstrated in human metastatic carcinoma cells (32) or in mouse keratinocytes in culture (12), eukaryotic cells in which 3 different types of IFs are coexpressed have not yet been reported excluding HSG cells used for the present study, as far as we know. However, we must stress that the 3 IF systems were not observed simultaneously in the same cell in HSG cells tested. The HSG cells which were cultured at either 34°C or 37°C were found to express 3 different types of IFs together, although desmin-type IFs alone disappeared in the cultivation of HSG cells at 39°C. This may indicate that the expression of desmin in the HSG cells proliferating in vitro is temperature dependent, although the possibility that desmin is degraded in the cultured HSG cells at 39°C and cannot be detected under the present experimental conditions is not excluded.

In HSG cells proliferating in vitro at certain conditioned temperatures, the expression of actin, tropomyosin, CEA, LF, and SC was observed, although the occurrence of myosin and S-100 protein in the cultured HSG cells was never detected under the present experimental conditions. The presence or absence of the cytoskeletal proteins such as actin and tropomyosin in the cultured HSG cells at different temperatures strongly suggests that the change of cellular morphology accompanying alterations of the microfilamentary system within the HSG cells occurs according to various cultivation temperatures. It has been described that the presence of CEA in human salivary gland neoplasms is related mainly to the well-differentiated ductal tissue in the neoplasms (29). Moreover, Caselitz et al. (7) have recently identified the occurrence of CEA in the acinar and intercalated duct cells of the normal human parotid gland. Since HSG cells have a fine structure similar to that of intercalated duct cells (38), the expression of CEA in the HSG cells strongly suggests that the HSG cells are of the intercalated duct type. Recently, the presence of LF has been demonstrated in the normal human salivary gland (33) and in certain human salivary gland neoplasms including the benign and malignant tumors (8). This LF can be considered to be related to the glandular function of the normal tissue, and an elevation of LF in saliva frequently occurs during the course of chronic recurrent parotitis (40). On the other hand, it is well known that human salivary glands contain IgA-producing cells and shed naturally IgA or SC into saliva (5). In addition, several investigators have reported the synthesis of SC in human tumors such as mammary, colonic, and pulmonary neoplasms (16, 19, 23, 31). However, Rognum et al. (34) have reported that the malignant alterations in large bowel adenomas impair the capacity of the columnar epithelium to produce SC and to express it as a functional receptor mediating uptake of dimeric IgA. The above findings about CEA, LF, and SC strongly suggest that the neoplastic intercalated duct cell line HSG with functional similarity to that of the normal duct cells is well differentiated.

On the other hand, the expression of IFs in the primary tumors arising in the inoculation site of HSG cells was essentially restricted to keratin- and desmin-type IFs, whereas coexpression of keratin-, vimentin-, and desmin-type IFs was observed in some neoplastic cells present in metastatic tumors in the regional lymph nodes and lung. In addition, the expressions of the cytoskeletal proteins such as actin, myosin, and tropomyosin and of S-100 protein were found in some neoplastic cells present in the metastatic tumors, although myosin and S-100 protein were not detected in the primary tumors. Recently, we have demonstrated the presence of myosin and S-100 protein in a neoplastic myoepithelial cell line of human salivary gland origin, but not in the duct-type HSG cells (35). In addition, Archer and Kao (1) also have identified immunohistochemically the presence of actomyosin in myoepithelium of human tissue. Moreover, a cell transitional between duct-type and myoepithelial cells has been demonstrated in the salivary gland tumor (24). This suggests a possibility of mutual conversion between duct-type and myoepithelial cells. Thus, the neoplastic cells containing myosin, S-100 protein, or desmin can be considered to represent exclusively the phenotype of myoepithelial cell lineage. This may indicate that some steps of differentiation towards the myoepithelial cells of the duct-type HSG cells can be represented in the process of metastasis in nude mice. Therefore, we now examine whether the neoplastic cells with an ultrastructure like myoepithelial cells or a cell type intermediate between the duct-type and myoepithelial cells are present in the metastatic tumors. Sheets of the neoplastic cells in the tissue sections from metastatic tumors of the regional lymph nodes and lung were almost occupied by the neoplastic cells showing oncocytic change, although these cells were not choked with many mitochondria as observed in the oncocytomas reported by Balogh and Roth (2), and Tandler et al. (41). Oncocytic change was not found in the cultured HSG cells at the 3 different temperatures for 3 days and in the primary HSG tumors. This morphological observation probably indicates that oncocytic cells in the salivary gland arise from the intercalated duct cells. Moreover, based on the above findings about the specific markers of neoplastic cells present in the primary and metastatic tumors, it can be considered that other differentiation stages different from the original HSG cells are induced during the course of metastasis in nude mice.
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Fig. 1. Detection of 3 different types of IFS in the cultured HSG cells at 37°C by the immunoperoxidase method, using anti-keratin (a), anti-vimentin (b), and anti-desmin (c), and anti-keratin (d), anti-vimentin (e) and anti-desmin (f), which were absorbed with the preparation enriched in IFS from cultured HSG cells. Hematoxylin counterstain. a to f, x 480. Note the mutual exclusiveness of the reactions with these sera on frozen tissue sections from the normal human oral tissues. Anti-keratin stained only the epithelium (g), whereas anti-vimentin (h) and anti-desmin (i) stained exclusively submucosal mesenchymal tissues and muscles, respectively. Hematoxylin counterstain. g, h, x 120; i, x 360.
Fig. 2. Immunohistochemical detection of the cytoskeletal proteins and specific markers in the cultured HSG cells at different temperatures. a, actin (39°C); b, tropomyosin (34°C); c, CEA (37°C); d, LF (37°C); e, SC (37°C). Arrows in d and e, positive staining. Hematoxylin counterstain, × 480.
Fig. 3. Indirect immunofluorescence microscopy of cultured HSG cells at 37°C. Double label immunofluorographs showing the simultaneous presence of keratin (a) and vimentin (b), of keratin (c) and desmin (d), or of vimentin (e) and desmin (f). First antibodies used: a, c, mouse monoclonal anti-keratin IgG; b, rabbit anti-vimentin; d, f, rabbit anti-desmin; e, mouse monoclonal anti-vimentin IgM. Second antibodies used: a, c, fluorescein-conjugated anti-mouse IgG; b, d, f, rhodamine-conjugated anti-rabbit IgG; e, fluorescein-conjugated anti-mouse IgM. × 640.
IFs AND SPECIFIC MARKERS OF SALIVARY ADENOCARCINOMA CELLS

Fig. 5. Histological appearance of the primary and metastatic tumors, which were produced by inoculation of HSG cells into nude mice. a, primary tumor. This is interpreted as adenocarcinoma with a solid and trabecular pattern. Anisocytism and mitosis are occasionally observed. H & E, × 240. b, photomicrograph demonstrating invasion of the skin covering the primary tumors. H & E, × 120. c, photomicrograph demonstrating the presence of neoplastic cells in vessels adjacent to the primary tumor bed (arrows). H & E, × 120. d, representative histological appearance of metastasis in the axillary lymph nodes. Oncocytic change is observed in many neoplastic cells. H & E, × 360. e, ultrastructure of the metastatic tumor formed in the axillary lymph nodes. Numerous mitochondria with various sizes and shapes are observed in the cytoplasm of neoplastic cells. × 15,000. f, representative histological appearance of metastasis in the lung. H & E, × 120. Black arrow, neoplastic cell nests; white arrow, bronchiolus; arrowhead, pulmonary alveolus.
Fig. 6. Immunohistochemical detection of IFs in the primary and metastatic nude mice tumors. Primary tumors: a, anti-keratin (positive); b, anti-vimentin (negative); c, anti-desmin (positive). Hematoxylin counterstain, a to c, ×360. Metastatic tumors in the lung: d, anti-keratin (positive); e, anti-vimentin (positive); f, anti-desmin (positive). Hematoxylin counterstain, d to f, ×360. The tissue sections from metastatic tumors present in the regional lymph nodes showed the positive stainings for keratin, vimentin, and desmin in a similar fashion as observed in the metastatic tumors in the lung (data not shown).
Fig. 7. Immunohistochemical detection of specific markers in the primary and metastatic nude mice tumors. Primary tumors: a, actin (positive); b, myosin (negative); c, tropomyosin (positive); d, CEA (positive); e, S-100 protein (negative). Hematoxylin counterstain. a to e, × 360. Metastatic tumors in the lung: f, actin (positive); g, myosin (positive, arrows); h, tropomyosin (positive); i, CEA (negative); j, S-100 protein (positive). Hematoxylin counterstain. f to j, × 360. The tissue sections from metastatic tumors present in the regional lymph nodes showed the positive stainings for the other specific antigens excluding CEA, in a fashion similar to that observed in the metastatic tumors in the lung (data not shown).
Intermediate-sized Filaments and Specific Markers in a Human Salivary Gland Adenocarcinoma Cell Line and Its Nude Mouse Tumors

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