Chapter 9

Salivary Gland Tumors

Mitsunobu Sato
Second Department of Oral and Maxillofacial Surgery, Tokushima University School of Denistry, 3 Kuramoto-cho, Tokushima 770, Japan. Tel: 0081-886-33-7353; Fax: 0081-886-33-7462; E-mail: satomitu@dent.tokushima-u.ac.jp

It has been proposed that tumors of the salivary gland arise from the intercalated duct cells, which can differentiate into both myoepithelial cells and acinar cells (1-3). In order to examine this hypothesis, a neoplastic intercalated duct cell line HSG, established from a human submandibular gland, has been used as a model for studying mechanisms regulating cytodifferentiation. In 1977, HSG cells were established from the submandibular gland resected from a patient who had received therapeutic irradiation for carcinoma of the floor of the mouth (4). Irradiation to the salivary gland causes acute lethal damage to various types of epithelial cell, including acinar, striated, and intercalated duct cells, and disturbs the glandular structure. Although this degenerative change persists without repair for a long period, intercalated duct cells can proliferate excessively among the damaged epithelia (5,6). This proliferation of surviving intercalated duct cells may be associated with the appearance of salivary gland tumors.

In 1987, two sublines (HSG-AZA1 and HSG-AZA3) were cloned after treatment of HSG with 5-azacytidine (7). HSG-AZA1 cells exhibit a phenotype similar to that of myoepithelial cells, including microfibrils and myosin expression, and form a myoepithelioma on transplantation into athymic nude mice. HSG-AZA3 cells have a phenotype similar to that of acinar cells, including cytoplasmic expression of secretory granules containing salivary amylase, and xenotransplantation of HSG-AZA3 cells results in the production of acinar cell carcinoma. This differentiation indicates that the neoplastic human salivary intercalated duct cell line HSG can differentiate into both myoepithelial cells and acinar cells. In addition, HSG-AZA11 cells,

J.R.W. Masters and B. Palsson (eds.), Human Cell Culture Vol. 1, 161–178.

which have a neuron-like cell phenotype, were cloned from 5-azacytidine-treated HSG cells in a similar manner (8).

In 1987, the parotid gland adenocarcinoma cell line HSY was established from an explant culture of a xenograft in a nude mouse (9). HSY cells have an ultrastructure similar to that of human salivary intercalated duct cells and express both amylase and vasoactive intestinal polypeptide (VIP).

In 1986, a neoplastic epithelial cell line, TYS, was isolated from an explant culture of a well-differentiated squamous cell carcinoma expressing carcino-embryonic antigen (CEA) that arose in human oral mucosa (10). Expression of CEA and amylase as well as ample tonofilaments were detected in cultured TYS cells. Transplantation of the cells into athymic nude mice resulted in the development of adenosquamous cell carcinoma containing CEA and amylase. Cultivation of TYS cells in the presence of sodium butyrate resulted in suppression of cell growth and production of secretory granules with human salivary amylase in the cytoplasm of the cells. When the sodium butyrate-treated cells were transplanted into athymic nude mice, a small mass developed transiently at the inoculation site and then disappeared. A biopsy of this mass was histopathologically interpreted as acinic cell carcinoma with squamoid features. These findings suggest that we have established a human adenosquamous cell line, presumably derived from a minor salivary gland present in oral mucosa.

In 1993, immortalized normal human salivary gland cells were established (11). Primary cultures of human submandibular gland cells were transfected with origin-defective mutant DNA of SV40. Using limiting dilution, 4 cell clones (NS-SV-DC, NS-SV-MC, NS-SV-SC, NS-SV-AC) with distinct morphologies were isolated. NS-SV-DC, NS-SV-MC, NS-SV-AC and NS-SV-SC have characteristics of duct, myoepithelial, acinar and squamous cells, respectively. Integration and expression of SV40 DNA were confirmed by Southern blot and indirect immunofluorescence staining. The DNA fingerprint analysis showed that the banding patterns of the 4 cell clones are identical, indicating that these cell clones are derived from a single donor. Anchorage-independent growth in semisolid agar and tumorigenicity in athymic nude mice were not observed and therefore the clones are considered to be immortal, but non-neoplastic.

Three cell lines (ACCS, ACCY and ACCAY) were isolated from three individuals with salivary adenoid cystic carcinoma (12,13). These cell lines form pseudocysts, which are a specific architectural feature of adenoid cystic carcinoma, and can produce abundant extracellular matrix including basal lamina components such as fibronectin, laminin, type IV collagen and glycosaminoglycans.

A salivary myoepithelial cell line UNC4 was isolated from a human salivary pleomorphic adenoma (14). UNC4 cells exhibit unique chromosome rearrangement and mucin production.

1. CULTURE CONDITIONS

HSG cells were established from explant culture of irradiated submandibular gland, grown in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum and 2mM L-glutamine. Epithelioid cells were harvested by treatment with 0.08% trypsin and 1.4% ethylenediamine tetraacetic acid (EDTA) in calcium- and magnesium-free phosphate-buffered saline (PBS(-); pH7.2). The single cell suspension was seeded into a 100-mm plastic Petri dish at a density of $3x10^6$ cells in 15 ml of growth medium. The cells were passaged at 5–6 day intervals. At the third passage the colony forming ability in semisolid agar was 9%. Suspensions of 100 or 1000 cells in growth medium containing 0.3% Special Agar Noble (Difco Laboratories, Detroit, Mich) were poured into agar medium thickened by the addition of 0.6% agar. After about 20 days' incubation, colonies were isolated with Pasteur pipets, and the cells were cultured in 3 ml of growth medium in 30-mm plastic Petri dishes until confluent cell monolayers were formed. A total of six clones were isolated from the colonies and examined using electron microscopy. These cells showed characteristics of intercalated duct-type cells of human salivary gland. The clone which grew best was designated HSG. Currently, HSG cells are cultured in MEM supplemented with 10% newborn calf serum and 2mM L-glutamine at 37°C in a 5% CO_2 incubator. HSG cells continue to grow logarithmically in the serum-free medium for three days after plating, although the number of HSG cells gradually decreases after four days of incubation.

HSG-AZAl and HSG-AZA3 cells were cloned as follows. Briefly, HSG cells (10^6 cells/dish) were seeded in 60-mm plastic Petri dishes in MEM supplemented with 10% newborn calf serum and 2mM L-glutamine. After 24h, 5-azacytidine was added at a concentration of 5 μM. The HSG cells were cultured in the continued presence of 5-azacytidine for 5 days, changing the growth medium daily. Then the treated cells were subcultured once in the growth medium without 5-azacytidine and cells were cloned using the colony-forming technique in semisolid agar medium as described above. The 7 subclones isolated were classified morphologically into 2 groups. Two subclones were composed of spindle-shaped or stellate cells and 5 were composed of polygonal cells with numerous secretory granules in the cytoplasm, whereas the parental HSG clonal cells were cuboidal and conical, grew with occasional tubular arrangement, and eventually formed multilayered foci. The subclones which showed the most stable growth in each of the 2 groups were designated HSG-AZA1 and HSG-AZA3, respectively. In another experiment, HSG-AZA11 cells were isolated from 5-azacytidine-treated HSG cells (8). HSG-AZA1 cells express a myoepithelial cell phenotype, HSG-AZA3 cells an acinar cell phenotype and HSG-AZA11 cells a neuron-like cell phenotype. The cells are cultured in MEM supplemented with 10% newborn calf serum

Table 1 Characterization of human salivary cell lines

Cell line	Patient age/sex	TNM category	Path stage and grade	Primary site	Specimen site	Culture method	Description	Differentiation	primary reference
HSG	54/male			submandibular gland	irradiated salivary gland	explant culture of operation material	salivary intercalated duct cell[4,12] expression of carcinoembryonic antigen(CEA), secretory component and lactofenin[12]	Differentiation into myoepithelial, acinar, keratinizing squamous, neuronal and smooth muscle cells	(4)
HSG-AZA1 (HSG-AZA1, HSG-AZA3 and HSG-AZA11 cells were cloned in vitro from the 5-azacytidine-treated HSG cells.)							DNA methylation level of HSG-AZA1, HSG-AZA3 and HSG-AZA1 1 cells significantly decreased when compared with HSG cells	Differentiation into neuronal cells	(7)
							HSG, HSG-AZA1 and HSG-AZA11 possesses the same restriction fragment length polymorphisms (RFLP)[8]		
							salivary myoepithelial cell[7]		
							expression of myosin, microfibrils with focal condensation and pinocytic vesicles[7]		

Continued on next page

Table 1 (continued)

Cell line	Patient age/sex	TNM category	Path stage and grade	Primary site	Specimen site	Culture method	Description	Differentiation	Primary reference
HSG-AZA3							expression of amylase[7]	Differentiation into chondrocyte, osteoblast, and keratinizing squamous cells	(7)
HSG-AZAl1							induction of synaptophysin and neuron specific enolase [8]	Formation of neurite-like structures	(8)
HSY	51/female	T2N0M0	Stage 2 grade2	parotid gland	primary tumor	explant culture of nude mouse-grown tumor	Amylase isozyme analysis salivary type expression of amylase and VIP[9]	Differentiation into neuronal cells	(9)
TYS	81/female	T2N1M0	stage 3 grade 1	minor salivary gland	primary tumor	explant culture of biopsy material	Amylase isozyme analysis salivary type[10] expression of CEA and amylase	Differentiation into acinar cells	(10)

Continued on next page

Table 1 (continued)

Cell line	Patient age/sex	TNM category	Path stage and grade	Primary site	Specimen site	Culture method	Description	Differentiation	Primary reference
NS-SV-DC	35/male			submandibular gland	nomaL salivary gland	explant culture of operation material	NS-SV-DC, NC-SV-MC, NS-SV-SC and NC-SV-AC possess the same RFLP and integrate SV40 DNAL[11]	Morphogenesis of human salivary gland13)	(11)
NS-SV-DC, NS-SV-MC, NS-SV-SC NS -SV-AC were cloned from the primary culture of human salivary gland which was transfected with origin-defective mutant DNA of SV40.									
							salivary intercalated duct cell[11]		
							expression of secretorycomponent[11]		
NS-SV-MC							salivary myoepithelial expression of myosin, myofilaments and pinocytic vesicles[11]		
NS-SV-AC							salivary acinar cell[11]		
							expression of amylase[11]		
NS/SV-SC							squamouscell[11]		
							expression of tonofilaments, desmosomes and 68kD cytokeratin[11]		

Continued on next page

Table 1 (continued)

Cell line	Patient age/sex	TNM category	Path stage and grade	Primary site	Specimen site	Culture method	Description	Differentiation	Primary reference
ACCS	56/female			maxillary sinus	primary sinus	explant culture of biopsy material	expression of cytokeratin, vimentin, S-100 protein and lactoferrin synthesis of type IV collagen and glycosaminoglycans	production of extracellular matrix including basal lamina components	(12)
ACCY	54/male			tongue	primary tumor	explant culture of biopsy material	expression of cytokeratin, vimentin, S-100 protein and lysozyme synthesis of type IV collagen and glycosaminoglycans	production of extracellular matrix including basal lamina components	(12)
ACCAY	63/female			submandibular gland	primary tumor	explant culture of operation material	expression of type IV collagen, fibronection, laminin, chondroitin 6-sulfate proteoglycan and heparin sulfate	production of basement membrane components	(13)
UNC4	51/female			parapharynx	primary tumor	in vitro culture of operation material	salivary myoepithelial cell expression of cytokeratin desmin, and epithelial membrane antigen	genetic rearrangement and salivary mucin production	(14)

Table 2 Tumor pathology and in vitro features of neoplastic and immortalized human salivary gland cell lines

Cell line	Original tumor pathology	In vitro features of cell line	Xenograft pathology
HSG	Irradiated submandibular gland. Parenchyma damage, but no neoplastic appearance, is visible.	Cultured HSG cells form multilayed foci with occasional tubular arrangement and indicate anchorage-independent growth.	Adenocarcinoma with solid and trabecular pattern
		HSG cells have ultrastructure of salivary intercalated duct cells; presence of desmosomes, tight junctions and intercellular digitations formed by papillary infoldings of the cytoplasmic processes.	Ultrastructurally, junctional complexes between neighboring cells represent desmosomes, tight junctions and intercellular digitations.
		The cytoplasmic organelles are often situated in one side of the cytoplasm.	
		HSG cells proliferated in the sponge matrix forming scattered cords with tubular structure.	
HSG-AZA1		HSG-AZA1 cells are spindle or stellate in shape and exhibit phenotypes similar to human salivary myoepithelial cells, such as microfibrils and myosin.	Myoepithelioma HSG-AZA1 nude mouse tumor cells have ultrastructure of salivary myoepithelial cells; e.g. presence of ample microfibrils with some areas of focal condensation in parallel fashion and pinocytic vesicles as well as microfilament systems which react positively with anti-myosin serum.

Continued on next page

Table 2 (continued)

Cell line	Original tumor pathology	In vitro features of cell line	Xenograft pathology
HSG-AZA3		Cultured HSG-AZA3 cells are composed of polygonal cells with numerous secretory granules in the cytoplasm and contain salivary amylase that is specific to salivary acinar cells.	Acinic cell carcinoma HSG-AZA3 nude mouse tumor cells have ultrastructure of human salivary acinar cells; e.g. presence of abundant secretory granules which react positively to anti-amylase serum.
HSG-AZA11		Cultivation of HSG-AZA11 cells in the presence of dibutyryl cAMP results in formation of neurite-like structures and induction of synaptophysin and neuron-specific enolase.	
HSY	Adenocarcinoma with solid and trabecular pattern. The tumor is composed of polygonal cells with a pale-staining eosinophilic cytoplasm, which are arranged to form ducts or tubules surrounded by hyalin-like substances or fibrous tissues.	Cultured HSY cells are characterized by large epithelial cells with large oval nucleus and reticulated cytoplasm as well as by formation of multilayered foci. Ultrastructurally, the profiles of cytoplasmic organelles in HSY cells resemble those in tumor cells in the original tumor or xenograft	Adenocarcinoma with solid and trabecular pattern Ultrastructure of xenograft is similar to that of the original tumor.

Continued on next page

Table 2 (continued)

Cell line	Original tumor pathology	In vitro features of cell line	Xenograft pathology
	Ultrastructurally, intercalated duct-type cells are found mainly in the tumor tissue; e.g. presence of desmosomes, tight junctions and intercellular digitations formed by papillary infoldings of the cytoplasmic processes.	HSY cells express VIP and amylase.	
TYS	Well-differentiated squamous cell carcinoma expressing CEA.	Cultured TYS cells are polygonal in shape, and grow in a pavement-like formation with occasional multilayered foci.	Adenosquamous cell carcinoma Keratinizing squamous cells and adenoid structure
		Almost all of the TYS cell population show strongly positive staining for CEA in the cytoplasm.	
		Ultrastructurally, TYS cells show desmosomes, tight junctions, ample microvilli as well as well-developed tonofilaments and secretory granules.	When TYS cells treated with sodium butyrate are transplanted into athymic nude mice, the tumor formed is interpreted as acinic cell carcinoma containing a squamous cell area
		Ultrastructure of the cultured TYS cells treated with sodium butyrate shows ample tonofilament, numerous secretory granules or vacuoles reactive to anti amylase serum and presence of the substance reactive to anti-CEA serum in the rims of vacuoles	

Continued on next page

Table 2 (continued)

Cell line	Original tumor pathology	In vitro features of cell line	Xenograft pathology
NS-SV-DC	Normal submandibular gland	NS-SV-DC cells show round- or polygonal-shaped morphology and duct-like structure, and express secretory component. NS-SV-DC cells seeded on Matrigel form round clusters consisting of refractile cells, and gradually slough off the Matrigel surface. Ultrastructurally, NS-SV-DC cells have intercellular digitations formed by papillary infoldings of the cytoplasmic processes.	Co-injection of NS-SV-DC cells with Matrigel into athymic nude mouse results in formation of cord similar to a duct-like structure.
NS-SV-MC		NS-SV-MC cells exhibit spindle-shaped morphology, and express myosin. NS-SV-MC cells seeded on Matrigel penetrate into the Matrigel. Ultrastructurally, NS-SV-MC cells have myofilaments and pinocytic vesicles.	Co-injection of NS-SV-MC cells with Matrigel into athymic nude mouse results in formation of amorphous structure with area of pericellular lysis.
NS-SV-AC		NS-SV-AC cells show polygonal-shaped morphology with numerous secretory granules, and express amylase.	

Continued on next page

Table 2 (continued)

Cell line	Original tumor pathology	In vitro features of cell line	Xenograft pathology
NS-SV-SC		NS-SV-SC cells exhibit flattened-shaped morphology, and have tonofilaments and desmosomal junctions as well as 68kD cytokeratin.	
ACCS	Adenoid cystic carcinoma with a cribriform and solid pattern	formation of cyst-like acellular space presence of ample extracellular matrix and microfibrils with occasional aggregation	no tumor formation
ACCY	Adenoid cystic carcinoma with a cribriform and trabecular pattern	formation of cyst-like acellular space presence of ample extracellular matrix and microfibrils with occasional aggregation	no tumor formation
ACCAY	Adenoid cystic carcinoma with a cribriform pattern	formation of cyst-like acellular space	no tumor formation
UNC4	pleomorphic adenoma	Butyrate or DMSO stimulation of UNC4 cultures results in upregulation of mucin production. When UNC4 cells are cultured on Matrigel, the cells grow in organized clumps and ductules within the substance of the matrix.	not determined

Table 3 Genetic changes of human salivary tumor cell lines

Cell Line	Chromosome	P53	oncogene
HSG	range from 49 to 72 (average: 66.3) including XY: triploid range (49-69)	wild type	expression of H-ras gene
HSG-AZA1	range from 60 to 79 (average: 66.7) including XU, triploid range (60-72)	wild type	expression of H-ras gene
HSG-AZA3	range from 63 to 76 (average: 67.8) including XY; triploid range (63-72)	wild type	expression of H-ras gene
HSY	range from 56 to 128 (average 81.3) including XX; triploid range (58-63)	not determined	not determined
TYS	not determined	mutant type[39]) (codon 281$^{Asp\text{-}His}$)	not determined
ACCS	range from 54 to 63 (average:59)	not determined	not determined
ACCY	range from 47 to 56 (average:50)	not determined	not determined
ACCAY	range from 43 to 46 + marker chromosome	not determined	not determined
UNC4	rearrangement between one chromosome 8 and the two chromosome 9s involving the 8q12 locus	not determined	not determined

and 2mM L-glutamine. These cells can grow logarithmically in the serum-free medium for at least 4 days.

HSY cells were established from explants of xenografts of a human parotid gland adenocarcinoma. TYS cells were established from explants of biopsy material obtained from primary tumor. Both HSY and TYS cells were cloned in a similar manner as follows. The fragments of tumor tissue were cultured in MEM supplemented with 10% newborn calf serum and 2mM L-glutamine. Explants with outgrowths of epithelioid cells alone were selected. When a monolayer of epithelioid cells was formed, the cells were harvested by treatment with 0.08% trypsin and 1.4% EDTA in PBS (-). These cells, dispersed in a single-cell suspension, were seeded into a 100-mm plastic Petri dish at a density of 3 million cells in 15ml of growth medium. Of colonies isolated at random, those that showed the fastest growth were designated HSY and TYS. Subculturing is done at 4-5 day intervals.

Immortalized human salivary gland cells (NS-SV-DC, NS-SV-MC, NS-SV-SC, NS-SV-AC) were established. Culture of normal human submandibular gland showing no pathological change, obtained during radical surgery for the treatment of submandibular sialolithiasis under general anaesthesia, was performed as follows: salivary gland specimens obtained at the operation

were immediately cut into approximately lmm³ explants and placed (approximately 10 explants/60-mm plastic Petri dish) onto type 1 pig tendon collagen gel-coated plastic dishes. Collagen gels were prepared according to the recommendation of the manufacturer (Nitta Gelatin, Osaka, Japan) and pre-equilibrated in growth medium for at least 24 hours before use. The growth medium employed in this study was SFM (GIBCO BRL, New York, NY) supplemented with 5ng/ml of recombinant epidermal growth factor (GIBCO) (SFM+). Cultures were grown in gassed, humidified incubators and the medium was changed every 2 to 3 days. When cells reached confluence, they were subcultured using 0.1% trypsin and 1.4% EDTA in PBS(-). Normal human salivary gland cells cultured in SFM+ were transfected using a liposome-mediated method. The SV40 ori-mutant DNA (obtained from the Japanese Cancer Research Resources Gene Bank, Tokyo, Japan) is a hybrid DNA consisting of plasmid (pMK16) and the full genome of SV40 DNA less 6 nucleotides at the Bg1I site. Transfection procedures were performed according to the recommendation of the manufacturer (GIBCO) with a minor modification. In brief, cells plated on collagen gel-coated dishes at 80 to 85% confluence were incubated for 24 h with lipofectin reagent-DNA complex, containing 10μg of SV40 ori-mutant DNA and 40 μg of lipofectin reagent. Approximately 2-3 weeks later, colonies were picked using a cloning syringe and reseeded on collagen gel-coated dishes in SFM+. Cloning of cells was by the limiting dilution method. Four cell clones showing cuboidal (NS-SV-DC), spindle (NS-SV-MC), flattened (NS-SV-SC) and polygonal (NS-SV-AC) morphology were established. Characterization of cell clones by ultrastructural examination and expression of specific antigens showed the similarity of NS-SV-DC, NS-SV-MC, NS-SV-AC and NS-SV-SC to duct, myoepithelial, acinar, and squamous cells, respectively.

ACCS, ACCY and ACCAY cells were established from explants of biopsy or resected primary tumor. These cell lines were isolated from outgrowths consisting only of epithelioid cells, grown in Dulbecco's modified MEM or MEM supplemented with 10% fetal bovine serum and 2mM L-glutamine.

UNC4 cells were established by culturing the cell suspension prepared from primary tumor. The cells were cultured in a base keratinocyte medium (GIBCO) supplemented with nonessential amino acids (0. 1mmol/L), glutamine (0.2mmol/L), insulin, hydrocortisone and transferrin (10mg/ml each), and epidermal growth factor (5ng/ml) without serum supplementation.

2. PATHOLOGY

HSG and its derivatives (HSG-AZA1 and HSG-AZA3) can differentiate into myoepithelial, acinar or neuronal cells as well as keratinizing squamous cells, chondrocytes, osteoblasts and smooth muscle cells, with a concomitant

decrease of anchorage-independent and anchorage-dependent growth and decreased tumorigenicity in athymic nude mice. HSG cells can differentiate into myoepithelial cells (HSG-AZA1), acinar cells (HSG-AZA3) or neuronal cells (HSG-AZA11) in response to 5-azacytidine (7,8), into myoepithelial cells in response to dibutyryl cAMP (15) or sodium butyrate (16), into keratinizing squamous cells in response to retinoic acid (17), into smooth muscle cells in response to etoposide (18), and into neuronal cells in response to epidermal growth factor (19). In addition, treatment of HSG-AZA1 cells with nerve growth factor (20), protein kinase inhibitor H-7 (21), or dibutyryl cAMP (22) results in their differentiation into neuronal cells. HSG-AZA3 cells can differentiate into chondrocytes in response to 22-oxa- 1α,25-dihydroxyvitamin D3 (23) and into keratinizing squamous cells in response to dibutyryl cAMP (24). When HSG-AZA3 cells are treated with 22-oxa-1α, 25-dihydroxyvitamin D3 in the presence of β-glycerophosphate, the treated cells exhibit enhanced expression of osteopontin and osteonectin mRNA and formation of bone nodules (25). When the tumors produced by transplantation into athymic nude mice of HSG-AZA3 cells are treated with 22-oxa-1α,25-dihydroxyvitamin D3, growth is significantly suppressed and bone formation is induced in the treated tumor. The tumor cells around bone express human osteopontin and osteonectin mRNA, detected by in situ hybridization. These findings indicate that the emergence of osteoblasts in the HSG-AZA3 cells occurs in the presence of 22-oxa- 1α,25-dihydroxyvitamin D3 and β-glycerophosphate.

It has been found that the HSG cell line and its derivatives HSG-AZA1, HSG-AZA3 and HSG-AZA11 express neurofilaments (Mr 200,000, 160,000, and 68,000) and specific antigens such as tubulin α and β chain, HNK-1 antigen and laminin, and stain with Bodian impregnation (7,8), the binding site for which is located in the b domain in the extended tail segment of neurofilament polypeptides (26). In addition, the findings that the HSG-AZA11 cells treated with dibutyryl cAMP form neurite-like structures and express synaptophysin indicate that the parental HSG cells may be neuroectodermal in origin (8). Moreover, the human parotid adenocarcinoma cell line HSY, cultured in the presence of dibutyryl cAMP, expresses some antigens specific to neuronal cells, such as a triplet of neurofilament polypeptides, neuron-specific enolase, synaptophysin, α or β-chains of tubulin, and HNK-1 antigen, whereas these antigens are not detected in the untreated cells (27). Furthermore, it has been found that HSG cells express intermediate-sized filaments such as cytokeratin, vimentin and desmin (28) as well as epidermal growth factor and transforming growth factor-β (29).

HSG cells form a glandular morphology and differentiate into acinar cells when cultured on the reconstituted basement membrane, Matrigel, which contains primary laminin as well as type IV collagen, heparin sulfate proteoglycan, and a number of growth factors such as epidermal growth factor,

fibroblast growth factor, and transforming growth factor-β. Laminin, one of the main components of Matrigel, is the major initiation factor in the differentiation of these cells (30). When SV40-immortalized cell clones with duct (NS-SV-DC) or myoepithelial phenotype (NS-SV-MC) are seeded on Matrigel in serum-free culture conditions, they fail to develop a morphogenesis consistent with salivary gland. On the other hand, three-dimensional morphogenesis of cells co-injected with Matrigel into the backs of athymic nude mice results in the characteristic features of each cell clone (31). NS-SV-DC cells align themselves into a cord, which is similar to the duct-like structure observed in normal salivary gland in vivo (32), whereas NS-SV-MC cells form an amorphous structure consisting of cuboidal and spindle-shaped cells, similar to the histologic appearance of a neoplastic myoepithelial cell line grown in the backs of athymic nude mice (33). The net balance of proteolytic activity is important in morphogenesis (34,35).

An in vitro system has been established in which conversion from non-metastasizing to metastasizing human adenocarcinoma cells can be induced (36). A human salivary adenocarcinoma cell clone HSGc with no metastatic ability, which was cloned from HSG cells, was exposed to N-methyl-N-nitrosourea (MNU). Following exposure to MNU, cells with altered morphology were cloned, which exhibited a flattened morphology with cytoplasmic processes, whereas HSGc cells were cuboidal or round. Following subcutaneous inoculation into athymic nude mice, MNU-treated HSGc clones formed metastatic foci in various organs, including lung, liver, spleen, pancreas and lymph node. Five metastasizing clones were isolated. The tumors growing at the inoculation site were diagnosed as adenocarcinoma with a solid and trabecular pattern, while the metastasizing clones produced tumors at the inoculation site which were classified as highly invasive clear-cell trabecular adenocarcinoma. Emergence of a clear-cell variant has been documented in a benign mixed tumor which recurred as a trabecular adenocarcinoma with clear-cell pattern (37). MNU-treated HSGc and metastasizing cell clones all metastasized to axillary and/or inguinal lymph nodes at a frequency of 100%. DNA fingerprint analysis confirmed that the metastasizing cell clones were of HSGc origin (36).

MNU-treated HSGc and metastasizing clones were found to secrete high levels of tissue-type plasminogen activator (PA), while HSGc produced undetectable levels of this enzyme. Expression of urokinase-type plasminogen activator (uPA) was not observed in any of the cell clones. Metastasizing clones produced higher levels of 57-and 32-kD, but not of 92- or 72-kD gelatinases, as compared to HSGc cells. Although tissue inhibitor of metalloproteinases-1 (TIMP-1) was detected in all cell clones, metastatic clones secreted less TIMP-1 than HSGc cells. These findings suggest that the acquisition of metastatic ability by HSGc cells is closely associated with increased secretion of several metalloproteinases as well as decreased or

altered TIMP- 1 expression. In addition, Northern blot analysis showed that a small amount of transforming growth factor-β receptor-I1 mRNA was detectable in HSGc cells, while no significant bands were detected in metastatic cell clones (38).

When xenografts of TY S were treated with a differentiation-inducing agent, vesnarinone [(3,4-dihydro-6-[4-(3,4)-dimethoxybenzoyl)- 1-piperaziny1]-2(1H)-quinolinone], given per os, a significant suppression of tumor growth was observed with tumor nests indicating keratinocyte and acinar cell differentiation (39). The tissue sections from vesnarinone-treated TYS tumors showed positive reaction with 3'-OH nick-end labelling and were stained strongly by monoclonal antibody (MAb) directed to carbohydrate antigen Le^Y, whereas the untreated tumors showed negative reaction with nick-end labelling and were infrequently stained by anti-Le^Y MAb. Within Le^Y-positive areas of the vesnarinone-treated tumors, keratinocyte and acinar cell differentiation as well as DNA fragmentation were frequently observed. It has been found that TYS cells have a mutant p53 gene at codon 281ASp-His (40), and that expression of $p21^{WAF1}$ and transforming growth factor-β (TGF-β1) mRNA is up-regulated by treating TYS cells with vesnarinone. In addition, treatment of TYS cells with vesnarinone resulted in the enhanced generation of latent TGF-β1 and the expression of TGF-β receptor (TβR), including TβR-I, TβR-II and TβR-III, was detected on TYS cells. These findings suggest that vesnarinone might directly induce expression of the $p21^{WAF1}$ gene in TYS, the product of which may be associated with the inhibition of cell growth and the induction of differentiation. Thus, TYS cells might be a model suitable for studying differentiation therapy of salivary gland cancer.

Detailed banding pattern analysis of UNC4 cells revealed rearrangements of chromosomes 8 and 9. The long arm of chromosome 8 is translocated to the long arm of chromosome 9. A distal segment of the long arm of chromosome 9 is translocated to the short arm of chromosome 9. A segment of the short arm of chromosome 9 is translocated to the truncated long arm of chromosome 8. It has recently been reported that salivary pleomorphic adenomas have structural chromosomal abnormalities including the following: translocations between chromosomes 3 and 8, chromosomes 6 and 16, chromosomes 8 and 9, chromosomes 8 and 12, chromosomes 8 and 14, and chromosomes 8 and 21 (41).

REFERENCES

1. Eversole, I.L. *Arch. Pathol.,* 92: 443, 1971.
2. Batsakis, J. G. *Oral Surg.,* 49: 229, 1980.
3. Pierce, G. B. *Am. J. Pathol.,* 77: 103, 1974.
4. Shirasuna, K., et al. *Cancer,* 48: 745, 1981.
5. Cherry, C. P., et al. *Br: J. Radiol.,* 32: 596, 1959.

6. Kashima, H. H., et al. *Am. J. Roentgenol.,* 94: 271, 1965.
7. Sato, M., et al. *Cancer Res.,* 47: 4453, 1987.
8. Sato, M., et al. *Cancer J.,* 6: 26, 1993.
9. Hayashi, Y., et al. *J. Nat. Cancer Inst.,* 79: 1025, 1987.
10. Yanagawa, T., et al. *Am. J. Pathol.,* 124: 496, 1986.
11. Azuma, M., et al. *Lab. Invest.,* 69: 24, 1993.
12. Shirasuna, K., et al. *Cancer Res.,* 50: 4139, 1990.
13. Sobue, M., et al. *Virchows Archiv B Cell Pathol.,* 57: 203, 1989.
14. Witsell, D. L., et al. *Arch Otolaryngol Head Neck Surg.,* 119: 1151, 1993.
15. Yoshida, Y., et al. *Cancer,* 57: 1011, 1986.
16. Azuma, M., et al. *Cancer Res.,* 46: 770, 1986.
17. Azuma, M., et al. *Cancer Res.,* 48: 7219, 1988.
18. Yoshida, H., et al. *Cancer J.,* 6: 220, 1988.
19. Aladib, W., et al. *Cancer Res.,* 50: 7650, 1990.
20. Iga, H., et al. *Cancer Res.,* 49: 6708, 1989.
21. Yoshida, H., et al. *Cancer J.,* 4: 267, 1991.
22. Kawamata, H., et al. *Cancer Invest.,* 10: 111, 1992.
23. Azuma, M., et al. *Cancer Res.,* 49: 5435, 1990.
24. Kawamata, H., et al. *Cancer J.,* 3: 274, 1990.
25. Sato, M., et al. *Cancer Lett.,* 115: 149, 1997.
26. Weber, K., et al. *Cold Spring Harbor Symp. Quant. Biol.,* 47: 717, 1983.
27. Nagamine, S., et al. *Cancer Res.,* 50: 6396, 1990.
28. Sato, M., et al. *Cancer Res.,* 45: 3878, 1985.
29. Sato, M., et al. *Cancer Res.,* 45: 6160, 1985.
30. Royce, L. S., et al. *Differentiation,* 52: 247, 1993.
31. Azuma, M., et al. *Lab. Invest.,* 70: 217, 1994.
32. Martinez-Madrigal, F., et al. *Am. J. Surg. Pathol.,* 13, 879, 1989.
33. Shirasuna, K., et al. *Cancer,* 45: 297, 1980.
34. Bacharach, E et al. *Proc Natl Acad Sci USA,* 89: 10686, 1992.
35. Montesano, R., et al. *Cell,* 62: 435, 1990.
36. Azuma, M., et al. *Int. J. Cancer;* 54: 669, 1993.
37. Evance, R. W., et al. In: Epithelial tumors of the salivary glands, pp276-277, Saunders, Philadelphia, 1970.
38. Azuma, M., et al. *Int. J. Cancer,* 68: 802, 1996.
39. Sato, M., et al. *Cancer Lett.,* 91: 1, 1995.
40. Sato, M., et al. *Cancer Lett.,* 112: 181, 1997.
41. Mark, H. F. L., et al. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.,* 82: 187, 1996.