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Long-Term Cultures of Salivary Gland Cells: Immortalization Versus Other Techniques (43782C)

KEDAR N. PRASAD[1]

Center for Vitamins and Cancer Research, Department of Radiology, School of Medicine, University of Colorado, Health Sciences Center, Denver, Colorado 80262–0278

A defect in saliva secretion is observed in human diseases such as Sjogren's disease. It is also observed during radiation therapy of cancer of head and neck when the parotid glands get incidentally irradiated. Therefore, the understanding of the mechanisms of regulation of the synthesis and secretion of salivary gland proteins is important. However, the direct investigation of molecular mechanisms that regulate the synthesis and secretion of salivary gland proteins has been hindered by the fact that long-term cultures of acinar cells have not been available. Also, the regulatory mechanisms of growth, differentiation, and transformation of acinar cells cannot be investigated until such cultures are established. Recent advances in tissue culture technology utilizing different approaches have made it possible to establish long-term cultures of epithelial cells including acinar cells from normal rat and human salivary glands, and human pleomorphic adenoma and adenocarcinoma. These approaches include: (i) immortalization of salivary gland cells by inserting T antigen genes from SV40 or polyoma viruses into the genome of these cells; (ii) maintenance and growth of epithelial cells from human pleomorphic adenoma and adenocarcinoma on a special substrate; (iii) maintenance and growth of acinar cells from normal adult salivary gland in specific serum free growth media; and (iv) maintenance of cells from parotid glands of transgenic animals that are programmed to develop salivary gland tumors. The purpose of this review is to critically analyze the potential usefulness and limitations of immortalized cell lines which have been generated by insertion of exogenous genes and compare them with those which have been produced by other methods.

Immortalization of Rat Salivary Gland Acinar Cells by Inserting T Antigen Genes

The acinar cells in adult rat salivary glands infrequently divide. They synthesize, store, and secrete salivary gland proteins following sympathetic and parasympathetic stimulation. During recent years, significant progress has been made in establishing the amino acid sequences and cDNA probes of several major salivary gland proteins (1–10) due to the availability of the tools of molecular biology and the large amounts of tissue that can be obtained from whole glands. The direct effects on receptor- and nonreceptor-mediated molecular events that regulate the synthesis and secretion of proteins in acinar cells cannot be studied *in vivo*. This, in part, may be due to the fact that some receptors which are located on sympathetic and parasympathetic cells are also present in acinar cells; therefore, it is not possible to distinguish between the effects which are produced by nerve stimulation and those produced by stimulation of receptors on acinar cells. The acinar cells in primary cultures grow slowly, rapidly lose differentiated functions, and die (11–14). Therefore, they are not suitable to study the molecular mechanisms which regulate the synthesis and secretion of salivary gland proteins. In addi-

[1] To whom requests for reprints should be addressed at Center for Vitamins and Cancer Research, UCHSC, Department of Radiology, School of Medicine, 4200 East 9th Avenue, Denver, CO 80262–0278.

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Table I. Available Cell Lines from Rat and Human Salivary Glands

Cell lines	Origin	Methods used for generation
2RS, uncloned	Rat parotid	Insertion of T antigen gene
2RSG, cloned	Rat parotid	Insertion of T antigen gene
2HP-C8, cloned	Human parotid, pleomorphic adenoma	Specialized substrate and growth medium
$2HP_1$, cloned	2HP-C8	
RSMT-A5, cloned	Rat submandibular	Methylcholanthrene treatment
HSG	Human duct cells, adenocarcinoma	Growth medium
Primary cultures	Human parotid	Specialized substrate and growth medium
Primary cultures	Rat parotid	Specialized substrate and growth medium

tion, they may contain a heterogeneous cell population, and this heterogeneity may increase as a function of time. Such cultures may not be suitable for biochemical or molecular analysis which requires large amounts of acinar cells. If long-term primary cultures of acinar cells, which divide frequently, can be established, they would be very useful for the study of regulation of growth and differentiated functions. The establishment of immortalized clonal cell lines of acinar cells can provide another tool to study some aspects of control mechanisms of growth, differentiation, and transformation. We have reported the establishment of immortalized clonal cell lines from adult rat parotid gland by inserting T antigen genes into acinar cell DNA (15). The plasmid vectors, pSV_3neo or pSV_5neo (16) were effective in producing immortalized acinar cells. The electroporation technique (17) was more efficient than calcium-phosphate-co-DNA precipitation technique (18) for a successful transfection of cells with plasmid vectors. Since the acinar cells in adult rat parotid divide infrequently, it was essential to induce cell-proliferation by injecting isoproterenol (15). A modified MCDB153 medium with supplemented growth factors and 10% heat-inactivated fetal calf serum (19) and tissue culture dishes with a special substrate (20) were found to be essential for the initial growth of transfected cells (15). At a later time, immortalized cells showed degenerative changes in the initial growth medium, which were reversible after the addition of RPMI medium with 10% fetal calf serum (15). These cells, at a later time, did not require a special substrate for their growth.

One of the immortalized cell lines which was isolated from cells transfected with pSV_5neo contained cells which appeared fibroblastic; however, when they reached confluency many of them matured to acinar-like cells which become large and filled with numerous granules. This cell line was eventually lost. The second cell line (2RS), derived from cells transfected with pSV_3neo, has been characterized in detail. Cells grew with a doubling time of about 25 hr, and did not produce tumors when injected subcutaneously into syngeneic rats or athymic mice (15). They produced T

Table II. Usefulness and Limitations of Available Cell Lines from Salivary Glands

Cell lines	Usefulness	Limitations
2RSG, cloned, nontumorigenic, acinar cells	Unlimited availability of homogenous acinar cells. Transcriptional controls of growth, differentiation and transformation	Insertion of T antigen gene may alter normal regulatory mechanisms
2HP-C8, cloned nontumorigenic, acinar cells	In addition to the above feature, comparative acinar functions between rat and human	Spontaneous genetic changes may have altered normal regulatory mechanisms
$2HP_1$, tumorigenic, acinar cells	Transcriptional control of malignancy, unique features of cancer cells	May not be adequate for normal regulatory mechanisms
Primary cultures, acinar cells	Regulatory mechanisms of differentiated functions within certain time of culturing	Limited tissue availability, heterogeneity, loss of differentiated functions
RSMT-A5, cloned, not well defined with respect to acinar functions	Control of malignancy, study of nonacinar functions	May not be adequate for normal regulatory mechanisms
HSG, tumorigenic ductal cells	Control of malignancy, study of nonacinar functions	May not be adequate for normal regulatory mechanisms

antigens in the nuclei, suggesting that T antigen genes have incorporated into DNA of immortalized cells, and that the insertion of T antigen genes is responsible for immortalization. They contained α-amylase as shown by positive immunostaining of cells and by dot-immunoblotting with the primary antibody to α-amylase. The activity of α-amylase in 2RS cell line as determined by substrate hydrolysis was very low (0.32 + 0.1 nmol/sec/mg protein) in comparison to rat parotid glands (11 70 + 30 nmol/sec/mg protein). The cells contain α-amylase specific mRNAs of 1176 and 702 bp which represent about 35% and 57% of those found in rat parotid glands. The low activity and level of α-amylase in the presence of relatively high level of (α-amylase mRNAs may be due to the following: (i) dividing acinar cells may lack storage granules for α-amylase, a characteristic feature of nondividing acinar cells in adult parotid glands; and (ii) dividing acinar cells translate α-amylase mRNAs at a reduced rate. We have isolated several clones from 2RS by a single-cell cloning method (15). Although the cell line (2RS) produces T antigens, it was never subjected to selection medium containing a lethal concentration of geneticin. When cells of 2RS line were grown in a selection medium containing a lethal dose of geneticin (400 μg/ml) for 2 weeks, many cells died. The survivors were recloned by a single-cell cloning method. One of them (2RSG) is being characterized in detail. The cells also contained T antigen, α-amylase, and α-amylase mRNA in the amount similar to those found in 2RS cell line. These cells had β_1 and β_2-adrenoceptors (unpublished observation).

The availability of immortalized clones of rat acinar cells provides a unique opportunity to investigate the molecular mechanisms which regulate the synthesis and secretion of α-amylase. The effects of receptor and nonreceptor-mediated cellular and molecular events can be investigated directly on acinar cells. The immortalized cell line may be most useful in the study of transcriptional control of salivary gland proteins since high levels of protein-specific mRNA are produced. This cell line may not be suitable for the study of regulation of leveling activity of protein, because these cells contain low levels of cell-specific proteins. In addition, the mechanisms of regulation of growth, differentiation, and transformation in acinar cells can be studied. The limitation of immortalized clones of acinar cells is that the inserted exogenous genes may destabilize genetic activity which regulates growth and cell-specific differentiated functions, and, therefore, may or may not represent regulatory mechanisms that are operating in normal acinar cells *in vivo*. The immortalized cells may spontaneously become transformed; therefore, it is essential that the tumorigenicity and certain cell-specific differentiated functions such as α-amylase be monitored at regular intervals in immortalized cells which are under investigation.

Immortalization of Human Parotid Acinar Cells

Our efforts to immortalize human parotid acinar cells by transfection of cells with plasmid vectors pSV_5neo or pSV_3neo have been unsuccessful. This may be due to the fact that the freshly prepared single-cell suspensions from normal human parotid glands contained very few dividing acinar cells and for successful transfection, a reasonable rate of cell proliferation is essential. Human parotid pleomorphic adenomas are generally considered benign; and therefore, may contain cells which have been spontaneously immortalized. They contain about 4% acinar cells, 62% ductal cells, 5% myoepithelial cells and, 3% other cells (21). This is in contrast to normal glands which contain 79% acinar cells, 3% ductal cells, 6% myoepithelial cells, and 12% other cells. The culturing and cloning of human pleomorphic parotid adenoma cells may yield immortalized acinar cells. Indeed, we have established two cell lines (2HP and $2HP_1$) from human pleomorphic adenoma (22). One of them (2HP) maintains epithelial cell morphology and is nontumorigenic, and the other ($2HP_1$) has lost epithelial morphology and is tumorigenic when tested in athymic mice. The $2HP_1$ line spontaneously emerged from the original cell line (2HP) after repeated passages for several months. These cells have lost epithelial morphology and contain small unipolar and bipolar cytoplasmic processes. Cells of both 2HP and $2HP_1$ exhibit α-amylase and α-amylase mRNA. They also contain neurofilament-160, a characteristic feature of nerve cells. This is a first demonstration that the human parotid acinar cell can have a neuronal specific marker. The significance of this observation is unknown. The levels of α-amylase specific mRNAs of 1176 and 702 bp represent 65% and 81% of those found in adult rat parotid glands, respectively. The activity of α-amylase in cultured cells ($2HP_1$) was low (0.32 nmole/sec/mg protein) in comparison to that which was obtained from the tumor (1.45 nmol/sec/mg protein) that was produced by the subcutaneous administration of the same cultured cells into athymic mice; however, it was similar to that found in the nontumorigenic cell line (2HP). The relatively high α-amylase activity in acinar cells growing *in vivo* in comparison to those grown *in vitro* may be due to the fact that acinar cells *in vivo* receive signals from sympathetic and parasympathetic systems which regulate the synthesis of α-amylase.

Immortalization and transformation are often used synonymously, but there is a clear distinction between them. The cells which divided *in vitro* as long as growth medium and space are available and are nontumorigenic, should be considered immortalized;

whereas the cells which divide *in vitro* like immortalized cells, but which form tumors in syngeneic animals or athymic mice, should be considered transformed. Thus, the cells of 2HP and $2HP_1$ are considered immortalized and transformed, respectively. Since both 2HP and $2HP_1$ cells contain similar activity of α-amylase, the low activity of α-amylase may be directly related to increased cell proliferation rather than transformation. The availability of a nontumorigenic clone (2HP-C8) and a tumorigenic clone ($2HP_1$) human acinar cells provides a unique opportunity to establish a biochemical distinction between tumorigenic and nontumorigenic cells, since both are derived from the same clone. In addition, a comparative study between human and rat non-tumorigenic acinar cells can be performed.

The cellular origin of human pleomorphic adenoma is controversial (21, 23–24). Some have suggested that pleomorphic adenomas are derived from myoepithelial cells (25), whereas others have disputed it (21, 23). Another hypothesis has proposed that pleomorphic adenomas are derived from primitive embryonal replacement epithelial cells (26). From this study it is not clear whether such primitive cells represent common precursor cells which give rise to various epithelial cell types or represent a precursor of specific cell types such as ductal, myoepithelial and acinar cells. We have proposed a hypothesis suggesting that the pleomorphic adenoma may arise by spontaneous transformation of a common precursor cell which maintains the capacity to differentiate into various cell types of parotid glands (22). Therefore, each of the cell types expresses its differentiated functions at low levels. This may, in part, account for the presence of different cell types in the pleomorphic adenomas. This observation may be analogous to neuroblastoma tumor which contains various neural cell types, and for which a similar hypothesis has been proposed (27).

Maintenance of Long-Term Cultures of Acinar Cells from Normal Rat Parotid Glands

Normal rat parotid acinar cells can be grown in culture for up to 6 months (28) under experimental conditions which were initially developed by Oliver *et al.* (29). These conditions involve tissue culture dishes coated with matrigel (1:1 dilution in F12), F12 medium supplemented with penicillin-streptomycin (100 unit/ml and 100 μg/ml); dexamethasone (10 μg/ml); insulin (5 μg/ml); transferrin (5 μg/ml); selenium (5 μg/ml); reduced glutathione (10 μg/ml); isoproterenol (1 μ*M*); putrescine (1 μ*M*); epidermal growth factor (10 μg/ml); and 10% heat inactivated rat serum. The addition of isoproterenol, epidermal growth factor, putrescine, insulin, and dexamethasone was considered essential for survival of acinar cells *in vitro*. The use of basement membrane matrix was also considered necessary. These cells exhibit both secretory granules and abundance of cellular organelles required for the synthesis, storage, and secretion of proteins. They also contain high levels of proline-rich protein mRNAs and proteins, and low levels of α-amylase-specific mRNAs and α-amylase (28). These results suggest that normal parotid acinar cells can be maintained in culture for a prolonged period of time with continued expressions of differentiated functions. The doubling time of these cells is not known. In addition, the life span of these cells has not been followed for a year or more.

The relative usefulness of clonal immortalized rat parotid cells developed by us (15) and primary cultures of normal rat parotid acinar cells established by others (28–29) for the study of molecular mechanisms of regulation and growth differentiation cannot be evaluated until these clones are characterized in detail with respect to receptor- and nonreceptor-mediated events that regulate the synthesis and secretion of proteins. At this time, immortalized cells (2RS or 2RSG) can be grown in regular tissue culture dishes without any special substrate. They grow very well in RPMI medium containing 10% fetal calf serum. No supplemental growth factors are needed for their growth and survival. They have not shown any evidence of transformation as yet.

Maintenance of Long-Term Cultures of Acinar Cells from Normal Adult Human Parotid Glands

Normal human epithelial cells *in vitro* exhibit a limited life span of three to five passages. A recent study has reported the establishment of a culture of acinar cells from normal human salivary glands. These cells grow with a doubling time of 42 hr and produce and secrete α-amylase by β-adrenoceptor-dependent mechanisms. These cells have been maintained for 35 passages (30). The success of culturing normal acinar cells was attributed to the following factors: (i) lack of serum in the medium; (ii) low concentration of Ca in the medium; (iii) low concentration of trypsin; and (iv) use of collagen as substrate for cells of late passages. Serum increases the growth of fibroblasts, but decreases the growth of normal epithelial cells. High Ca concentrations cause differentiation of epithelial cells which later die in culture. The use of low trypsin concentration during removal of cells reduced chemical trauma to cells. The cells from higher passages require collagen substrate for optimal attachment and growth. The omission of serum and reduction of calcium in media have also been useful in maintaining long-term cultures of epithelial cells of other tissues (20, 31–33). If the primary cultures of homogeneous acinar cells, such as that described above, can be established, then such cultures will be more suited to study the regula-

tion of synthesis and secretion of salivary gland proteins than the immortalized cell lines.

Sabatini *et al.* (34) have reported the establishment of 19 cell lines from human normal salivary glands and three from parotid pleomorphic adenomas. These cells can be serially cultivated on 3T3 feeder layer. The growth medium was a mixture of 3:1 (F12:DMEM) supplemented with 2.5% fetal bovine serum, 0.4 μg/ml hydrocortisone, 8.4 μg/ml cholera toxin 5 μg/ml insulin, 24 μg/ml adenine, 1.8 m*M* Ca^{++}, and 10 μg/ml epidermal growth factor. The continued proliferation of epithelial cells required the presence of a feeder layer, since these cells degenerated in regular tissue culture dishes after a limited number of cell divisions. The epithelial cells from human and macaque salivary gland and pleomorphic adenoma gland grew well under the above experimental conditions. At least some cell lines from normal parotid glands expressed varying levels of genes for two acinar cell-specific markers, α-amylase and proline-rich proteins at least during early passages up to five (34). These cell lines can also be used as tools for the study of mechanisms which regulate the expression of major salivary proteins. Since these cell lines were not followed for a longer period of time, it is not known whether they can maintain differentiated functions for longer periods.

Epithelial Cell Culture from Rat Submandibular Gland

RSMT-A5 cell line has been derived from rat submandibular gland after treatment with methylcholanthrene (35). These cells grow in McCoy's 5A medium containing 10% fetal bovine serum and 100 μg/ml each of penicillin G and streptomycin sulfate. Isoproterenol treatment increases cAMP level by 130-fold in these cells, suggesting the presence of β-adrenoceptor system. The presence of α-amylase or proline-rich proteins was not studied in this cell line. Stimulation of β-adrenoceptors causes an early and transient increase in the expression of c-*fos* protooncogene (36). A further study shows that the increase in c-*fos* expression is not related to proliferation of these epithelial cells (36). Since the presence of α-amylase or proline-rich proteins was not studied, it is not certain whether RSMT-A5 cells are acinar cells. Nevertheless, chemical carcinogen treatment can be used to generate transformed cell lines from salivary glands.

Cultures of Neoplastic Cell Lines from Human Adenocarcinoma

The human neoplastic salivary intercalated duct cells (HSG) produce tumors in athymic mice. TPA treatment of these mice increased the growth of transplanted tumor and induced α-amylase synthesis (37). This suggests that both intercalated and acinar cells may have a common precursor cell. Dibutyryl cAMP treatment causes reversible differentiation into myoepithelial cells (38). This cell line may serve as a useful tool to study the mechanisms which control the differentiation of precursor cells into acinar and nonacinar cells.

The treatment of neoplastic intercalated ductal cells (HSG) with 5 μ*M* 5-azacytidine for 5 days generated several clones. Two clones contained cells which were spindle-shaped and exhibited features of myoepithelial cells. Five clones contained cells which appeared polygonal and contained numerous secretory granules and α-amylase (39). Thus the neoplastic intercalated ductal cells can differentiate into acinar cells as well as myoepithelial cells by treatment with azacytidine. This further suggests that transformation initially may occur in common precursor cells which maintain the capacity to differentiate into various epithelial cell types in the salivary glands after an appropriate treatment. Thus, treatment of cells with chemical carcinogens can generate useful cell lines for the study of differentiation and transformation of salivary gland cells.

These studies reveal that normal, immortalized, and transformed clonal acinar cells from rodent and human salivary glands are now available (Table I). They should be useful in elucidating the receptor and nonreceptor-mediated molecular events which regulate the growth, differentiation, and transformation of acinar cells. In addition, the regulatory mechanisms of the synthesis and secretion of salivary gland proteins can now be investigated in a direct manner. The relative value of each cell line generated by different approaches cannot be evaluated at this time until these cell lines are fully characterized with respect to specific receptors, cell-specific markers, and regulatory mechanisms. The usefulness and limitations of each available cell line have been summarized in Table II.

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