### **Invited Review**

# Morphogenesis of normal human salivary gland cells *in vitro*

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Summary. Primary cultured human salivary gland cells were transfected with ori-defective mutant DNA of SV40. Following 2-3 weeks of transfection, slowly expanding colonies consisting of small compact cells emerged, while mock-transfected cells did not grow any more and eventually entered crisis, followed by cell death. Using limited dilution technique, we isolated 4 cell clones with distinct morphology from a single colony. Morphological observation of cells cultured on plastic dishes precisely revealed the characteristics of the constituent cells of salivary gland; i.e., three cell clones showing cuboidal- (NS-SV-DC), spindle- (NS-SV-MC), and flattened morphology (NS-SV-SC) were similar to duct-, myoepithelial-, and squamous phenotype, respectively. A remaining cell clone, polygonal in shape and with numerous secretory granules (NS-SV-AC), resembled an acinar cell.

Characterization of cell clones by ultrastructural examination and search for 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. Anchorage-independent growth in soft agar and tumorigenicity in nude mice were not recognized in all cell clones. These results demonstrate that establishment of cell clones with duct-, myoepithelial-, acinar-, or squamous phenotype was accomplished in the in vitro system, and that based on the evaluation of colony-forming ability in soft agar and tumorigenicity in nude mice these cell clones can be considered to be non-tumorigenic. Using the above in vitro system, we examined the effect of a reconstituted basement membrane extract, Matrigel, on the morphogenesis of cultured normal human salivary gland cells. When NS-SV-DC or NS-SV-MC were seeded on Matrigel in serum-free culture conditions, they formed round or zonal clusters on day 1; failing however, to develop into a salivary gland morphogenesis. Semithin sections of cell clones cultured on Matrigel exhibited multicellular aggregates on day 1, while on days 2 and 3 these cells lost both cell-cell and cell-Matrigel interactions and eventually entered crisis. In an attempt to understand the mechanism involved in this phenomenon, we have investigated proteolytic enzymes and their inhibitors secreted by cell clones. Although cell clones produced almost identical levels of gelatinases, they released increased amounts of plasminogen activators (PAs) as compared with a neoplastic human salivary gland cell line (HSG), which had already been demonstrated to differentiate into acinar cells when cultured on Matrigel. Obvious difference of expression level of tissue inhibitor of metalloproteinases-1 (TIMP-1) was not observed in these cells. However, secretion of PA inhibitors was elevated in NS-SV-MC when compared to NS-SV-DC. Neutralization of excess PAs by exogenously-added serine protease inhibitors corrected the aberrant morphogenesis of NS-SV-DC, but not that of NS-SV-MC, and allowed NS-SV-DC to form glandular-like structures. Thus, these results may suggest that a tightly regulated proteolytic activity is, in part, essential for in vitro morphogenesis of normal salivary gland duct cells.

**Key words:** Human salivary gland, Morphogenesis, Matrigel, Proteolytic enzymes

### Introduction

The morphogenetic development of human salivary gland is initiated from the floor of the mouth as a budding of the epithelium into a closely associated mesenchyme, followed by the formation of the stalk proximally and the rounded bud distally, that finally results in the establishment of highly branched secretory ducts and acini (Bernfield and Banerjee, 1982; Bernfield et al., 1984). During these developmental processes, the interactions between the epithelium and mesenchyme lead to the stimulation of epithelial cell proliferation and production of matrix components such as type I collagen and fibronectin by mesenchymal cells (Banerjee et al.,

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1977; Cohn et al., 1977), which contributes to the stabilization of multilobular structures of salivary gland. Also, the epithelium itself produces the basement membrane components, including type IV collagen and laminin, to separate the epithelial buds and stalks from the surrounding mesenchyme, which also supports the characteristic morphology of salivary gland. Thus, establishment of normal lobular morphology and cytoarchitecture of salivary gland is fully dependent on the integrity of extracellular matrices including the basement membrane (Bernfield et al., 1972).

Recently, increasing evidence indicates that a reconstituted basement membrane, Matrigel, can profoundly influence cell growth, metabolism, and differentiation in vitro in a number of cell types (Kleinman et al., 1981; Gospadorowicz, 1984; Hay, 1984; Reid and Jefferson, 1984; Kibbey et al., 1992; Royce et al., 1993). For example, in vitro neoplastic human salivary gland cells have been shown to form glandular-like morphology and differentiate into acinar cells when cultured on Matrigel (Kibbey et al., 1992; Royce et al., 1993). In addition, laminin has been identified one of the main components of Matrigel, as the major initiating factor in the differentiation of these cells (Kibbey et al., 1992). Indeed, these observations have clearly demonstrated the significance of the basement membrane in the morphogenesis of in vitro salivary gland cells with neoplastic phenotype; however, it still remains uncertain how the morphogenetic development of normal human salivary gland cells is regulated by the basement membrane.

Thus, in this review we will describe some of the recent advances in our understanding on the morphogenesis of normal human salivary gland cells. These findings were elucidated by the analysis of SV40-immortalized normal human salivary gland cell clones, which had been established in an *in vitro* system in our laboratory (Azuma et al., 1993).

#### The constituent cells of human salivary glands

In human major salivary glands including parotid, submandibular, and sublingual glands, secretory units for the salivary secretion are composed of acini, and intercalated, striated and excretory ducts (Dardick et al., 1990). At a cellular level, mainly three distinct types of cells are recognized based on morphological and functional differences; acinar cells where saliva is produced, myoepithelial cells by which salivary secretion is promoted, and duct cells that carry the secretion to the oral cavity. In addition to these three cell types, ductal squamous cells, which shift to the oral squamous cells, are also observed at the distal portions of excretory ducts.

To date, we have demonstrated that when neoplastic human salivary gland cells with duct-, myoepithelial-, acinar-, or squamous-phenotype were cultured on plastic surfaces, morphological differences are clearly distinguishable among four types of cells (Azuma et al., 1986, 1988; Yoshida et al., 1986; Sato et al., 1987); cells with cuboidal, spindle, and flattened morphology, respectively, correspond to duct, myoepithelial, and squamous cells. In addition, polygonal-shaped cells with numerous secretory granules are similar to acinar cells. Accordingly, the above observations elucidated by the analysis of a neoplastic cell line prompt us to assume the working hypothesis that the constituent cells of normal human salivary gland might be established as cell lines in culture by means of immortalization techniques such as transfection or infection of viral DNA (Christian et al., 1987; Stoner et al., 1991; Willey et al., 1991).

### Immortalization of normal human salivary gland cells

Normal human salivary glands showing no pathological disorders were obtained at surgery, and immediately subjected to the primary culture. After 5 to 7 days of culture, favourable outgrowths of epithelial cells were observed only when tissues were cultured on type I collagen gel-coated dishes in serum-free keratinocyte medium (SFM). In addition, under the above conditions, growth of fibroblasts was selectively inhibited. Cells at 80 to 85% confluence were transfected with origin defective mutant DNA of SV40 (SV40 ori<sup>-</sup> mutant DNA) (Gluzman et al., 1980) using a liposome-mediated transfer method (Fraley et al., 1980; Schaefer-Ridder et al., 1982; Felgner et al., 1987; Willey et al., 1991). The SV40 ori<sup>-</sup> mutant DNA is a hybrid DNA consisting of plasmid (pMK16) and the full genome of SV40 DNA less 6 nucleotides at the BglI site (Gluzman et al., 1980). Approximately 2-3 weeks after the transfection, colonies that appeared in dishes were picked up by a cloning syringe and reseeded on collagen gel-coated dishes in SFM. When cells reached confluence, they were subcultured and divided into 2 groups; one seeded on collagen gel-coated dishes, and the other on plain plastic dishes. Interestingly, when SV40 DNA-transfected cells were cultured on plastic dishes, morphological differences were clearly distinguishable among cells. Thus, we obtained 4 morphologically distinct types of cell clones (NS-SV clones), and simultaneously they were referred to as NS-SV-DC, NS-SV-MC, NS-SV-AC, and NS-SV-SC (Fig. 1). Morphologically, NS-SV-DC, NS-SV-MC, and NS-SV-SC, exhibited cuboidal, spindle, and flattened shapes, respectively, and NS-SV-AC showed polygonalshaped morphology with numerous secretory granules. By ultrastructural examination and search for specific antigens of cell clones, NS-SV-DC, NS-SV-MC, NS-SV-AC, and NS-SV-SC were demonstrated to be similar to duct, myoepithelial, acinar, and squamous cells, respectively (Azuma et al., 1993). Since cell clones with an extended life did not enter a crisis phase as defined by quiescent or greatly reduced period of growth, that has been described for SV40-transformed cell types of both mesenchymal (Girardi et al., 1965; Sack, 1981) and epithelial (Kaighn et al., 1980; Chang et al., 1982; Taylor-Papadimitriou et al., 1982; Banks-Schlegel and

Howley, 1983; Steinberg and Defendi, 1983; Moyer and Aust, 1984) origin, these cell clones are considered to be apparently immortal cell lines.

# Anchorage-dependence, -independence, and tumorigenicity of NS-SV clones

NS-SV clones did not form anchorage-independent colonies in soft agar. This suggests that they are anchorage-dependent. The evaluation of anchoragedependent growth of cell clones on plastic dishes showed the colony-forming efficiency ranging from 2 to 13%. Although it is generally recognized that human epidermal cells require a layer of irradiated 3T3 cells as feeder cells for clonal growth (Rheinwald and Green, 1975), human urothelial cells infected with SV40 have been shown to be able to grow in the absence of 3T3 feeder cells, exhibiting loss of the requirement of feeder cells for clonal growth (Christian et al., 1987). In fact, the colony-forming efficiency of SV40-transfected epidermal and SV40-infected urothelial cells, respectively, ranged from 0.02 to 0.06% (Banks-Schlegel and Howley, 1983) and from 5 to 14% (Christian et al., 1987), whereas that of NS-SV clones was between 2 and 13%, indicating that the culture condition used is sufficient to maintain clonal growth of NS-SV cells. The tumorigenic potential of cell clones was tested by inoculating  $5-7 \times 10^6$  cells subcutaneously into the backs of nude mice and scoring the development of tumors. In animals with more than 5 months followup. no tumor formation was noted, suggesting that NS-SV clones were not tumorigenic in nude mice. These findings are in keeping with those previously obtained with immortalized cells (Garcia et al., 1986, 1991; Christian et al., 1987; Stoner et al., 1991). Thus, immortalization appears to be a discrete, intermediate, and necessary but not sufficient step between normal and malignant cell behaviour (Gaffney et al., 1970; Kaighn et al., 1989). This hypothesis is supported by the reported conversion of nontumorigenic immortalized human keratinocytes to tumorigenicity by incorporation of additional viral genes (Rhim et al., 1985) or treatment with chemical carcinogens (Rhim et al., 1986).

### Branching morphogenesis of the salivary gland

It is recognized that the morphogenesis of salivary gland is initiated from the floor of the mouth as a budding of the epithelium into a closely associated mesenchyme. This leads to the stimulation of epithelial cell proliferation and production of matrix components such as type I collagen and fibronectin by mesenchymal



Morphological appearance of cell clones isolated after transfection with SV40 orimutant DNA. When transfected cells were grown on plastic dishes, four morphologicallydistinct types of cells emerged. Cells with cuboidal (a), spindle (b), and flattened (d) morphology were similar to duct, myoepithelial, and squamous cells, respectively. Polygonal-shaped cells with numerous secretory granules (c) resembled acinar cells. Phase-

contrast microscope. x 200

Fig. 1.

cells (Spooner and Faubion, 1980; Bernfield et al., 1984), resulting in the stabilization of multilobular structures of salivary gland. Also, the epithelium produces the basement membrane components

comprising mainly type IV collagen and laminin separating the surrounding mesenchyme (Banerjee et al., 1977; Cohn et al., 1977). Thus, establishment of normal lobular morphology and cytoarchitecture of salivary



Fig. 2. Morphogenetic behaviour of cell clones cultured on Matrigel in SFM. NS-SV-DC (a to f) and NS-SV-MC (g to I) were observed at 24 hours (a, b, g, and h), 48 hours (c, d, i, and j), and 72 hours (e, f, k, and I) after seeding by phase-contrast microscope (a, c, e, g, i, and k) and light microscope (b, d, f, h, j, and I). At 24 hours, NS-SV-DC (a) and NS-SV-MC (g), respectively, formed round and zonal clusters consisting of cells with a refractile property. Semithin sections (b and h) revealed round cell aggregates. With time of culture, NS-SV-DC (c and e) lost cell-cell interactions and actually sloughed off the Matrigel surface (d and f). By 72 hours after seeding, NS-SV-MC (i and k) demonstrated the penetration of the Matrigel with areas of pericellular lysis (j and I). Mat: Matrigel layer; Med: medium layer. x 200

gland is fully dependent on the integrity of extracellular matrices (Bernfield et al., 1972). In addition, proteolytic enzymes secreted by both mesenchymal and epithelial cells also play an important role in the morphogenetic processes of salivary gland, particularly in the destruction of the basement membrane necessary for cell proliferation and development of new buds at the distal tips of the gland (Bernfield et al., 1984); the basement membrane on the distal lobules becomes incomplete, because of cell proliferation, by degrading type IV collagen and proteoglycan. Subsequently, after branching morphogenesis is nearly complete, the epithelium is again surrounded by a complete basement membrane. Thus, these findings suggest that remodelling of the basement membrane regulated by proteolytic enzymes and their inhibitors is a critical factor in salivary gland morphogenesis.

To date, accumulated evidence indicates that a



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reconstituted basement membrane, Matrigel, which contains primary laminin as well as type IV collagen, heparin sulphate proteoglycan, and a number of growth factors such as epidermal growth factor, fibroblast growth factor, and transforming growth factor-ß (Kleinman et al., 1982; McGuire and Seeds, 1989), can profoundly influence cell growth, metabolism, and differentiation *in vitro* in a number of cell types including salivary gland cells (Kleinman et al., 1981; Gospodarowicz, 1984; Hay, 1984; Kibbey et al., 1992), i.e., Kibbey et al. (1992) and Royce et al. (1993) have demonstrated that neoplastic human salivary gland cells form glandular-like morphology and differentiate into acinar cells when cultured on Matrigel, and that laminin, one of the main components of Matrigel, is the major initiating factor in the differentiation of these cells. Therefore, we attempted to examine the effect of Matrigel on the morphogenesis of normal human salivary gland cells, which had been established in an *in vitro* system by transfection with SV40 DNA in our laboratory.

## Morphogenetic development of SV40-immortalized normal human salivary glands cells

As shown in Fig. 2, when SV40-immortalized cell clones with duct- (NS-SV-DC) or myoepithelial phenotype (NS-SV-MC) were seeded on Matrigel in



Fig. 3. Morphogenetic behaviour of cell clones co-injected with Matrigel into nude mice. a. NS-SV-DC formed a duct-like structure showing an alignment of themselves into a cord by 3 weeks after inoculation into nude mice. b. an amorphous structure with areas of pericellular lysis was observed when NS-SV-MC were injected into nude mice. x 100

serum-free culture conditions, they formed round or zonal clusters on day 1; however, they failed to develop into a salivary gland morphogenesis. Semithin sections of cell clones cultured on Matrigel exhibited multicellular aggregates on day 1, whereas on days 2 and 3, these cells lost both cell-Matrigel and cell-cell interactions and eventually entered crisis. On the other hand, three-dimensional morphogenesis of cell clones, which was established by co-inoculation with Matrigel into the backs of nude mice, revealed characteristic

NS-SV-DC

features of each cell clone (Fig. 3); NS-SV-DC aligned themselves into a cord, which is similar to a duct-like structure observed in normal salivary gland *in vivo* (Martinez-Madrigal and Micheau, 1989). Another characteristic of NS-SV-DC is the formation of normal cell aggregates comprising three to five cells. These observations are in keeping with those previously described (Shirasuna et al., 1981), and these confirm the nature of NS-SV-DC to be either an intercalated or a striated duct cell. The histology of inoculum formed



by implantation of NS-SV-MC with Matrigel into nude mice was interpreted as an amorphous structure consisting of cuboidal and spindle-shaped cells, similar to the histological appearance of a neoplastic cell line grown in the backs of nude mice (Shirasuna et al., 1980). Although areas of pericellular lysis of Matrigel were commonly seen, in no instance did we observe the apparent cell death. Thus, the in vivo study may imply that the morphogenetic behaviour of each cell clone with each characteristic can be achieved by establishing some suitable culture conditions in our in vitro model system. Therefore, to identify the mechanism involved in the disruption of in vitro morphogenesis, we examined the proteolytic enzymes and their inhibitors secreted by cell clones. Because the net balance of proteolytic activity is documented to be an important factor for the development of physiological morphogenesis in various tissues and organs (Bernfield et al., 1984; Montesano et al., 1990; Bacharach et al., 1992). To investigate this possibility, we analyzed the expression levels of plasminogen activators (tissue-type plasminogen activator, tPA; urokinase-type plasminogen activator, uPA), collagenases, endothelial-type plasminogen activator inhibitor (PAI-1), and tissue inhibitor of metalloproteinases-1 (TIMP-1) secreted by cell clones. Since it is well known that PAs and collagenases, respectively, are neutralized by PAI-1 and TIMP-1, both proteases and their inhibitors are critical factors for the evaluation of the net enzyme activity.

Consequently, we found that NS-SV-DC and NS-SV-MC express consistently identical levels of collagenases and TIMP-1. The collagenolytic nature of NS-SV-DC and NS-SV-MC was the clearance of gelatin substrate at the molecular weights of approximately 100 and 68 kD and 66 and 57 kD, respectively. The characteristic of gelatinolytic feature of NS-SV-MC is the presence of 57 kD gelatinase. Based on the molecular weight, this gelatinase may be attributed to stromelysin (Korczak et al., 1991). On the contrary, cell clones secreted considerably higher levels of PAs than a neoplastic human salivary cancer cell line (HSG), which had already been demonstrated to form glandular-like structures when cultured on Matrigel (Royce et al., 1993). In addition, NS-SV-DC produced a relatively small amount of PAI-1 as compared with NS-SV-MC. Therefore, these findings indicate the net balance of a high serine protease activity in cell clones, particularly in NS-SV-DC. The finding that the proteolytic balance between PAs and PAI-1 is altered in NS-SV-DC and NS-SV-MC, as compared with that in HSG, raised a hypothesis that excessive proteolytic activity might be responsible for the aberrant morphogenetic behaviour of these cells. Thus, to clarify this hypothesis, we examined the behaviour of cell clones grown on Matrigel in the presence of exogenously-added inhibitors of serine protease, including soybean trypsin inhibitor (STI) and Trasylol. When STI was added to the culture system, the morphogenetic behaviour of NS-SV-DC was dramatically altered (Fig. 4). NS-SV-DC cultured on

Matrigel in the presence of STI formed, within 24 hours, glandular-like structures. These consisted of both elongated duct-like structures and multilobular cell clusters. Examination of the cultures with a high-power phase-contrast microscope revealed a similarity to the organization of intercalated ducts with secretory elements (Dardick et al., 1990). In addition, semithin sections of NS-SV-DC to which either STI or Trasylol was added confirmed that NS-SV-DC were organized as an excretory unit-like structure that consists of both acini and duct system. Similar results were obtained with NS-SV-DC cultured on Matrigel in the presence of Trasylol. Although the precise mechanism by which STI or Trasylol corrects the aberrant morphogenetic development of NS-SV-DC is not elucidated, it is suggested that STI or Trasylol exerts its effect by neutralizing PAs, resulting in the reduction of matrix degradation (Montesano et al., 1990).

In contrast to the observation with NS-SV-DC, STI and Trasylol at the concentrations used did not affect the morphogenetic behaviour of NS-SV-MC seeded on Matrigel. We have no precise idea concerning this phenomenon; however, it has been shown that myoepithelial cells present in mammary and salivary glands appear to be primarily responsible for the degradation of the basement membrane during normal developmental processes (Bernfield et al., 1984; Dickson and Warburton, 1992). For example, in the rat mammary gland, the increased expression of 72 kD gelatinase and stromelysin (molecular weight 57 kD) (Korczak et al., 1991), both of which are implicated in basement membrane degradation, is mostly restricted to myoepithelial cells. In addition, it is suggested that secretion of stromelysin correlates well with the proteolytic aggressiveness of transformed cell lines (Cajet et al., 1989; Matrisian et al., 1991; Sreenath et al., 1992). These findings, therefore, may indicate that stromelysin itself has an important role in the degradation of basement membrane components necessary during the morphogenetic development of mammary and salivary glands. The analysis of CM by gelatin-zymography clearly revealed that NS-SV-MC secrete two types of gelatinases, 66 kD gelatinase and stromelysin. Thus, the failure of restoration of aberrant morphogenetic behaviour of NS-SV-MC by addition of serine protease inhibitors to the culture system may be due in part to the production of stromelysin by NS-SV-MC.

### **Concluding remarks**

Cell clones with duct-, myoepithelial-, acinar-, or squamous phenotype of normal human salivary gland have been developed by the transfection technique with SV40 ori<sup>-</sup> mutant DNA. Importantly, NS-SV clones are not tumorigenic when injected into nude mice, nor could they form colonies in soft agar. Thus, they should serve as useful models to investigate the regulation of cell growth and differentiation of normal human salivary

glands. Based on the above consideration, morphogenetic development of NS-SV-DC and NS-SV-MC grown on Matrigel in serum-free culture conditions was investigated. The results indicate that neutralization of excess proteolytic activity by exogenously-added serine protease inhibitors corrected the aberrant morphogenetic development of NS-SV-DC but not of NS-SV-MC and was responsible for the formation of glandular-like structures by NS-SV-DC. These findings suggest that a tightly regulated proteolytic balance is critical for the morphogenesis of human salivary glands, and that an alteration of the balance results in the disruption of the normal morphogenetic development of duct cells. Although it is unknown whether the secretion of stromelysin by NS-SV-MC contributes to the disturbance of morphogenetic development on Matrigel and to the degradation of the basement membrane necessary for the normal developmental processes of human salivary glands, suppression of stromelysin by procedures such as treatment of NS-SV-MC with antistromelysin antibody might give us a clue to establish in vitro morphogenesis of myoepithelial cells on Matrigel.

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