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Use of Microgravity Bioreactors for Development of an In Vitro Rat Salivary Gland Cell Culture Model

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Abstract During development, salivary gland (SG) cells both secrete factors which modulate cellular behavior and express specific hormone receptors. Whether SG cell growth is modulated by an autocrine epidermal growth factor (EGF) receptor-mediated signal transduction pathway is not clearly understood. SG tissue is the synthesis site for functionally distinct products including growth factors, digestive enzymes, and homeostasis maintaining factors. Historically, SG cells have proven difficult to grow and may be only maintained as limited three-dimensional ductal-type structures in collagen gels or on reconstituted basement membrane gels. A novel approach to establishing primary rat SG cultures is use of microgravity bioreactors originally designed by NASA as low-shear culture systems for predicting cell growth and differentiation in the microgravity environment of space. These completely fluid-filled bioreactors, which are oriented horizontally and rotate, have proven advantageous for Earth-based culture of three-dimensional cell assemblies, tissue-like aggregates, and glandular structures. Use of microgravity bioreactors for establishing in vitro models to investigate steroid-mediated secretion of EGF by normal SG cells may also prove useful for the investigation of cancer and other salivary gland disorders. These microgravity bioreactors promise challenging opportunities for future applications in basic and applied cell research.

Key words: exocrine, endocrine, EGF, signal transduction, microgravity, cell culture

One of the most challenging tasks in the study of normal cellular processes and abnormal disease states is the development of cellular models that sufficiently mimic in vivo responses. For instance, an in vitro rat salivary gland (SG) model would permit investigation, at the cellular level, of disorders such as oral tumors, salivary dysfunction, Sjogren's syndrome, and latent virus infections. We are investigating the use of low turbulence microgravity bioreactors to establish rat SG tissue culture models for the study of signal transduction mechanisms and gene expression in response to steroid hormones and epidermal growth factor (EGF). Currently, no standardized technique for SG cell culture is available, and SG cells from mature animals are difficult to grow by conventional means. The clinostatic-like, fluid-filled microgravity bioreactors, designed at the NASA Johnson Space Center in Houston, are conducive to the three-dimensional growth of a number of primary and established cell types. Originally designed as the best way to predict the relative advantages of the microgravity environment of space for the culture of cells, these specialty bioreactors are laterally rotated, fluid-filled vessels with an internal membrane oxygenator. Figures 1 and 2 show two types of microgravity bioreactors used for three-dimensional modeling and culture of difficult-to-grow cell types in our laboratory. These 50, 100, and 250-mL-volume rotating-wall vessels, partially simulating microgravity hydrodynamics, have shown excellent performance in Earth-based cell tests. Research on microgravity bioreactor-grown SG cells can provide significant information on cell surface receptor and steroid hormone-mediated response of salivary gland cells, as well as facilitate the culture of these recalcitrant cells.

In the processes of cell and tissue differentiation, freedom of spatial orientation of cells and substrates is important for individual cell response to mechanical contact with other cells, as well as to soluble modulators. The low turbulence culture environment of the microgravity bioreactors is especially conducive to the formation of multidimensional, tissue-like assemblies

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of cells that can facilitate the study of inter- and intracellular mechanical and biochemical signaling.

Mechanisms by which hormones may influence temporal growth of cells of different tissue origin are not clearly understood. Development of an in vitro rat SG cell culture model system can provide an invaluable tool for investigating mechanisms of hormonally induced interrelationships between different types of tissues of endocrine and exocrine systems. There is a differential presence of specific estrogen receptors in salivary gland tissue of female rats in vivo and a time- and estrogen-related appearance of EGF receptors in uterine tissue. These observations are the basis for our investigation using surgically removed salivary glands from rats to determine whether the presence of estrogen receptors in the salivary gland cells may be functionally important to the estrogen-induced appearance of EGF receptors in uterine tissue in stimulating long-term tissue growth.

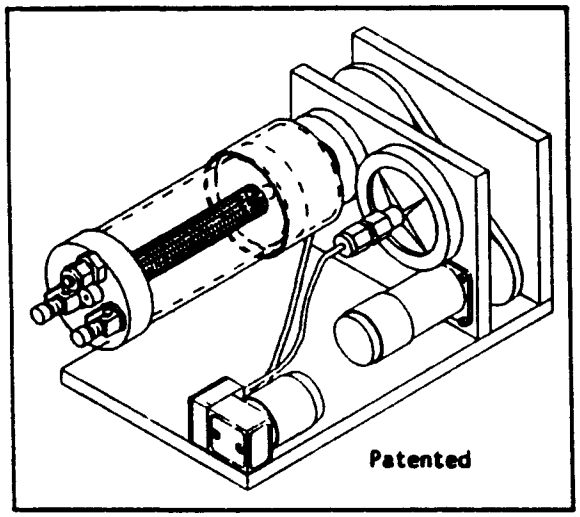

Fig. 1. A type of microgravity bioreactor, the Synthecon Rotary Cell Culture System (Synthecon, Inc , Friendswood, TX), consists of a vessel that rotates around its horizontal axis Cells are suspended individually or on microcarrier beads in culture medium that completely fills the chamber, and oxygen dissolves into the medium from air pumped through a hydrophobic membrane around the center post

Understanding the role of estrogen-mediated salivary gland EGF secretion in controlling endometrial tissue growth could possibly provide a link between salivary gland function and the development of neoplasias in endometrial or other EGF-mediated growth responsive tissue. An in vitro model for salivary gland secretion of EGF, which could be shown to be dependent on estrogen, would be an invaluable tool for investigating mechanisms for stimulating long-term tissue growth. This research is significant as ground-based research because it is expected to

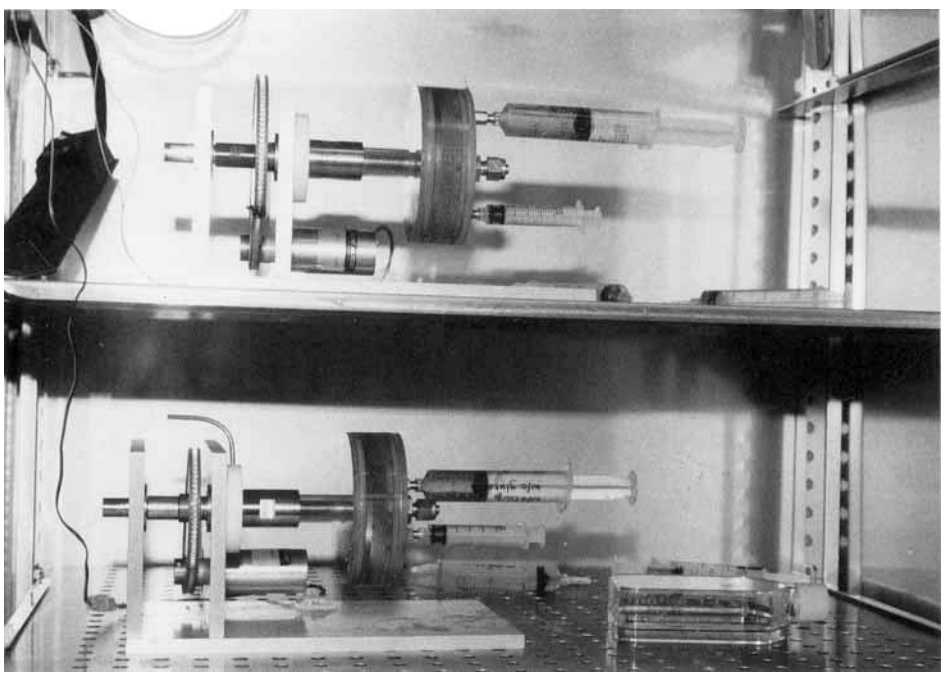

Fig. 2. A second type of microgravity bioreactor This is a simple batch-type system with a silicone membrane at one end to allow an oxygen and carbon dioxide exchange The systems are designed to operate in a standard CO_2 tissue culture incubator Samples are withdrawn and medium is replenished through syringe ports on the face of the vessels

provide basic information on how estrogen and androgen hormones mediate EGF synthesis and secretion by use of an in vitro model. The validated rat SG cell model and procedures can be applied to the culture of human SG tissue for the investigation of cancer, salivary gland dysfunction, virus infection, and basic cellular responses.

HISTORICAL PERSPECTIVES

Possible Regulation of Endocrine Systems by Exocrine Factors and Involvement of Epidermal Growth Factor

The regulation of endocrine systems by exocrine SG factors has not been well explored. That such interaction exists in vivo was documented [Boyer et al., 1986] for rats in which the submandibular salivary gland was removed (sialoadenectomy). This resulted in a small but significant decrease in testicular weight. Sialoadenectomy in female rats results in a slightly impaired uterine growth response to exogenous estradiol [Campbell, unpublished observations]. Campbell et al. [1990] also demonstrated differential presence of specific estrogen receptors in the SG tissue of female rats, and Kyakumoto et al. [1986] reported differences, based on the sex, in the distribution of androgen receptors in submandibular glands of mice. Mukku and Stancel [1985] reported an estrogen-related temporal appearance of EGF receptors in uterine tissue in vivo. Salivary gland ductal cell growth may be influenced by a mechanism that involves EGF per se, indicating an EGF signal transduction pathway. EGF may indeed be a required SG cell growth factor. Although SG cell growth and function may be modulated by an EGF receptor signal transduction pathway, this has not yet been characterized [Durban, 1990]. We are currently investigating the relationship of increased estrogen secretion during the estrus cycle in rats and concomitant salivary gland EGF secretion in vivo. The approach of using the microgravity bioreactors for the development of an in vitro model for in vivo response can provide a significant advancement to the knowledge of endocrine/exocrine regulation in an area in which reliable cell cultures are not readily available.

Bioreactors and Cell Culture

Although there are numerous bioreactors that grow cells efficiently for a multitude of purposes, most do not specifically provide extremely low-shear environments conducive to cell–cell association leading to tissue-like architecture. Bioreactors fall into three major categories [Griffiths, 1986]: a) those with medium flowing over the cells, such as the hollow fiber bioreactors; b) those with heterogeneous mixing, such as stack plate reactors, in which cells are grown on flat surfaces stacked within the vessel and medium is circulated around the stacks; and c) homogeneous mixing bioreactors, in which the cells are grown on microcarrier beads that are kept in suspension by some type of mixing device. A description of novel bioreactor systems, cell types cultured, products obtained, and advantages and disadvantages has been published previously [Margaritis and Wallace, 1984]. The hollow fiber and stack plate bioreactors are efficient for producing cell products such as monoclonal antibodies, but do not permit easy sampling of the cells or examination of the cell–cell interactions within the fibers or plates. In addition, the pores of the fibers may become plugged if medium flow rate is not optimized for product concentration and cell density. Recovery of the cells for further study is difficult.

The advantages of bioreactors that grow anchorage-dependent cells on microcarriers include potential for tissue modeling, scale-up, and a large surface area for cell attachment and growth with less space occupied per unit of culture surface, compared to two-dimensional monolayer cultures in flasks. In addition, growth can be monitored relatively easily by counting released nuclei [van Wezel, 1973]. A disadvantage to the culture of cells in most commercially available reservoir-type bioreactors is that mixing and mass transfer are achieved by various types of marine impellers, stirring bars, vibromixers and paddles [Croughan et al., 1987]. Damage to cells grown on microcarrier beads in conventional bioreactors with headspace and impellers has been attributed mostly to fluid mechanical forces [Cherry and Papoutsakis, 1988]. Using round-bottomed tissue culture reactors with agitation provided by impellers, and carbon dioxide and oxygen controlled by air flow through the headspace, Cherry and Papoutsakis described the mechanisms of damage to cells on microcarriers. Their data indicate that cell damage or death is a result of impeller collisions, simple collisions of beads against cells, turbulent eddies, and other fluid velocity gradients. Other limitations include cell death resulting

from the desiccation of cells trapped in bubbles when sparging is used as a means to maintain sufficient oxygen levels and suspend the cells. Then, obviously, only the most hearty cells could survive to lay down ECM and a basal lamina-type material, and thus, conventional bioreactors "select" against fragile or less hearty cell types. For this reason, resultant tissue assembly in vitro in conventional bioreactors is automatically limited. It is specifically this limitation that the microgravity bioreactors avoid by providing a low turbulence culture environment.

Microgravity and Microgravity Bioreactors

The microgravity bioreactors incorporate a combination of concepts (zero headspace, horizontal rotation, membrane oxygenation) to produce a NASA spinoff technology specialty bioreactor only recently available to the research community. In some respects, the rotating-wall, zero-headspace bioreactors appear to simulate the results achieved in microgravity. Morphological characteristics of cells from plant seedlings germinated during Space Shuttle flight were indistinguishable from those of cells germinated in a microgravity bioreactor [Moore, 1990]. Space-based research has demonstrated enhanced cell attachment to beads when primary human kidney cells and collagen-coated microcarrier beads were mixed in low-g and then fixed at designated times during Space Shuttle flight, STS-8. Based on cell counts and electron micrographs, it was evident that cells attached to bead surfaces more readily in microgravity than in the Earth-based static controls [Tschopp et al., 1984; Lewis et al., 1984].

The use of horizontally rotating culture systems, clinostats, for low-gravity simulation is well documented [Briegleb, 1983; Cogoli, 1987; Dedolph and Dipert, 1971]. Clinostats differ from the microgravity bioreactors described in this article mainly in the design of the bioreactor oxygenator system, which provides dissolved gases and allows long-term culture (weeks to several months) with appropriate medium changes, and the lack of air–liquid interface within the vessel. Numerical models of rotating bioreactors indicate a partial microgravity simulation with respect to the major trajectories and circulation time frame of a slowly sedimenting particle's motion. The fluid mechanical aspects of these bioreactors are addressed by Tsao et al. [1992]. Comparisons of trajectories of particles in unit gravity and microgravity indicated that a slowly sedimenting particle in unit gravity will follow the same major trajectory as that of a particle in microgravity, except for gravity-induced spatial oscillation on each revolution. Tsao reports that this is true even though the strengths of the dominant forces controlling the dynamics are quite different. The slow rotation of the microgravity bioreactors in unit gravity prevents sedimentation of particles to the bottom of the vessel and obviates the turbulence incurred from stirring devices in conventional bioreactors.

TABLE I. Types of Cells Grown in Microgravity Bioreactors

Anchorage dependent	Suspension cultures
BHK-21	Primary mouse bone marrow stem cells
Normal human keratinocytes	SP2 (mouse myeloma fusion partner)
Primary human embryonic kidney	L-1210 leukemic cells of mice
Primary rat osteoblasts	Mouse hybridoma cell lines
Mouse osteoblast cell line	Pancreatic tissue
Rat salivary gland fibroblasts	Human lymphocytes
Human neuroblastoma	
Human breast cancer	Plant cells (tobacco callus)
Human prostate cancer	
Human lung cancer	
Human melanoma	
Human kidney cancer	
Normal small intestine (epithelial and fibroblasts)	
Human skin fibroblasts	
Melanocytes	
Rat hepatoma	

The slowly rotating clinostatic-like microgravity bioreactors possibly provide the best way of achieving low turbulence, microgravity-like culture conditions on Earth. In a number of ground-based experiments, the clear advantage of these horizontally rotating culture systems for the development of three-dimensional cultures from both primary cells and established cell lines has been demonstrated [Lewis, 1990; Lewis et al., 1987; Lewis et al., 1989; Lewis et al., 1992; Lawless et al., 1989]. Table I lists some of the types of cells cultured in the microgravity bioreactors and Figure 3 shows a three-dimensional aggregate of mouse osteoblast 3T3 cells growing on microcarrier beads. These cells differentiated

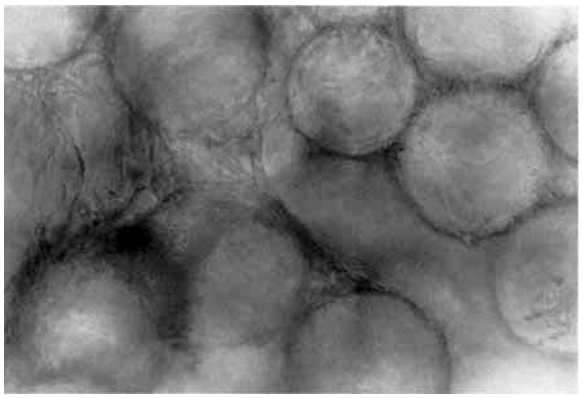

Fig. 3. Mouse osteoblast 3T3 cells (8-day culture) growing in three-dimensional aggregates of cells and microcarrier beads The cells formed a tissue-like structure between beads and secreted collagen into the matrix (shown by electron microscopy of 14-day samples)

and produced collagen fibrils in the matrix between the beads [Lewis et al., 1991]. It is extremely significant that this bone cell three-dimensional model clearly shows that basal lamina—in this case collagen—can form around the microcarriers. In the case of our SG cell model, formation of this type of basal material can function as a matrix for the association of glandular cells and provide secreted factors for their growth. Another model culture achieved in these bioreactors is three-dimensional, tumor-like aggregates of human colon cancer cells. These cultures appeared as differentiated, glandular structures that secreted mucsin [Goodwin et al., 1992]. Based on these results with varied types of cells, the microgravity bioreactors should enhance primary culture of rat SG cells and indeed, our preliminary tests have demonstrated feasibility.

ADVANTAGES OF MICROGRAVITY BIOREACTORS FOR DEVELOPING SALIVARY GLAND CELL CULTURE MODELS

SG cells are not easily cultured. Only cells from immature animals [Redman et al., 1988], those cultured in collagen gels [Durban, 1990], initiated as aggregates plus single cells [Kiser et al., 1990], or grown on reconstituted basement membranes [Oliver et al., 1987] grow well enough to study. SG cell cultures are usually maintained for only a short time and the cells do not differentiate or divide [Amsterdam and Jamieson, 1974; Barka and Van Dor Noen, 1975; Kanamura and Barka, 1975; Mangos et al., 1975]. Oliver [1980] reported the development of a nondividing acinar cell culture lasting for a month and the growth of exocrine acinar cells on a reconstituted basement membrane [Oliver et al., 1987]. Calcium and growth factor requirements for submandibular duct cell culture was described by Kurth et al. [1988], and culture of mouse submandibular cells and differentiation in long-term culture was achieved in collagen gels by Durban [1990]. EGF in combination with dexamethasone greatly increased survival time of rat SG cell cultures [Redman et al., 1988].

The most significant advantages of microgravity bioreactors for SG cell culture are low shear, reduced risk of cell damage, and increased opportunity for cell–cell interactions, so that all of the prerequisite cell types dissociated from the original SG tissue can reassemble to form a three-dimensional, tissue-like assembly. Because the various cell types attach to a bead substrate and communicate with one another by a complex series of events beginning with the laying down of extracellular matrix and a basement material, the subsequent release of various growth factors increases the chance of cellular growth in low turbulence systems. In normal cellular communication, intracellular signals transduced to and from the cell membrane induce differentiation and other cellular responses to external stimuli. Communication between different types of interacting cells can occur by way of the extracellular matrix (ECM). This matrix consists of materials that are secreted by cells into the intercellular spaces [Wessels, 1977]. The matrix frequently contains large concentrations of complex polysaccharides such as hyaluronic acid and glycosaminoglycans, which differ in different tissues. Linked to proteins, they form proteoglycans. These complex glycoproteins, including fibronectin, laminin, and collagen fibers, are laid down between the interacting cells; it is this basal lamina that is involved in the development and maintenance of glandular structures such as salivary glands. Salivary gland cells require time in culture to lay down basal lamina and for cells to interact prior to differentiating into glandular structures.

There are several well-known cell surface interactions with extra cellular matrix (ECM) leading to protein synthesis and secretion, but the mechanisms of signal transduction across the plasma membrane are not well understood. For instance, collagen gels are necessary for casein synthesis in mouse mammary epithelial cells

[Lee et al., 1984], and laminin-rich gels are necessary for the long-term secretion of albumin by rat hepatocytes [Bissell et al., 1982]. SG stromal cells may be expected to lay down adhesion factors, attach, and grow on the surfaces of collagen-coated beads. Three-dimensional glandular structures could then develop in association with these attached cells. At rotation rates sufficient to keep the cells and beads suspended (8–15 rpm), the cells do not collide with vessel walls and cell–bead collisions are of low impact. This gentle environment is expected to increase the success of SG cell culture in the microgravity bioreactors. Three-dimensional cell–bead aggregates are formed under these low-shear conditions when the cells, in monodispersed suspension with beads in the culture medium, bridge bead to bead. In conventional bioreactors with stirring devices, the degree to which aggregates increase in size depends on the rate of formation and the rate of destruction of the bridges [Cherry and Papoutsakis, 1988]. One way to decrease the destruction of bridges is to reduce fluid dynamic stress, a condition characteristic of the microgravity bioreactors. As the cells become confluent on the surfaces of the beads, larger assemblies of beads are bridged by cells that maintain the structural integrity of the assembled cell–bead system.

PRACTICAL CONSIDERATIONS

Clearly, the practical application of this cell culture technology must be demonstrated by a significant number of investigations with rat salivary gland tissue. Optimum concentration ranges for mediating hormones must be established from dose response studies. A major concern is the lack of a strongly reacting specific rat EGF receptor antibody. This will be a critical area for future research and development. With such an antibody, cell surface markers can be visualized and quantitated in response to various hormones and factors. The EGF content of cells and the secreted EGF in the culture medium will show the functional differentiation and response of SG cells to estrogen and androgen treatment.

In the area of molecular-level response after the binding of mediators to cell surface receptors, new information on the mechanics of signal transduction is emerging rapidly in the scientific literature. EGF is probably one of the most characterized polypeptide growth factors. It exerts its effect through plasma membrane receptors. The investigation of SG cell surface membranes should provide timely concepts for insights into early events after EGF binding. In most cells responsive to EGF, an early event after its binding to specific receptors on the plasma membrane is the breakdown of phosphatidylinositol-1,4,5-biphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). The binding of EGF leads to a series of rapidly occurring events involving tyrosine kinase and second messengers. In most cell types, after the initial series of reactions following EGF binding to the cell membrane, specific genes are switched on and may be detected by in situ hybridization. Probing for *c-myc*, *c-jun*, and *c-fos* protooncogene products will provide significant information on SG cell regulatory mechanisms and an assessment of the early signal transduction events mediated by EGF will help to characterize the responsiveness of the SG model. The possibility of receptor-mediated signal transduction mechanisms can be investigated by measuring EGF binding to SG cell surfaces.

The fundamental benefits of microgravity bioreactors for SG cell culture are just beginning to be realized. The limited information gained in our laboratory has led to encouraging prospects for the development of rat salivary gland cell models, and we have demonstrated the feasibility of culturing rat SG cells in the microgravity bioreactors. SG cells from female rats formed basal layers of fibroblast-like cells on surfaces of microcarrier beads. Within 7 days, nonattaching aggregates of large glandular-like cells associated as clusters between beads. This delicate association of beads and cells is expected to enhance the development of the rat SG cell model. In addition, preliminary investigations with chick pancreatic tissue have shown that cell aggregates can be maintained for several weeks in the microgravity bioreactors, and that cells remained functionally active and secreted significantly higher levels of amylase than did static culture controls [Conway et al., 1992].

FUTURE DIRECTIONS

Earth-Based Tissue Engineering

A new discipline in the area of cell culture is emerging. Tissue engineering is basically the growth of any human tissue or organ by the use of bioreactors to create three-dimensional cultures [Edgington, 1992]. Several biotechnology companies are currently working on processes

in which external forces may be used to control cells and their products, and possibly to confer differentiation ability. The specialty bioreactors, such as the microgravity system, may also have a potential use in organ replacement, grafting, and cartilage replacement. Skin cells grown in other systems are already being tissue-engineered for grafting.

Tissue modeling, architecture, and differentiation depend on the arrangement and juxtapositioning of interacting cells and on EGF- and ECM-mediated selective adhesion effects on cell shape and distribution. ECM and EGF also affect intracellular responses leading to specific gene activity and differentiation [Darnell et al., 1986]. Culture of cells in the horizontal, rotating fluid-filled bioreactors results in high-density cell growth and the organization of cells into uniform, multicellular aggregates with high cell viability [Lewis et al., 1992]. The same type of enhanced associations of the heterogeneous cell types derived from SG tissue are expected to lead to the differentiation and maintenance of in vivo-like function.

Novel Environment, New Directions, New Dimensions—Space-Based Research

Future research will not be limited to terrestrially cultured cells. A primary future direction is to gain basic information on EGF secretion and salivary gland physiology and function in the ground-based, in vitro model, rat salivary gland cell culture. The low-shear bioreactors will play a significant role in successfully establishing three-dimensional models to determine cell growth, differentiation, and function of SG tissue as a candidate for future spaceflight experiments to determine the role of gravity on cellular processes. Signal transduction, gene regulation, and cellular function in response to endocrine and exocrine factor mediation (EGF, androgen, and estrogen) will be significant mechanistic areas of research applicable to human space physiology. From information gained, a knowledge base sufficient to assess the scientific value of mammalian cell and tissue culture under low-gravity conditions, where mechanical stresses are reduced and can be controlled, will be the beginning of space-based cell culture technologies.

The utility of microgravity bioreactors for ground-based research lies in modeling a low-shear, altered-gravity environment in which to investigate cell growth and differentiation. Ground-based experiments in these bioreactors can establish the scientific foundations for future research directions in microgravity and provide an environment intermediate between unit gravity and space low-gravity. Results of spaceflight experiments with mammalian cells have demonstrated a profound effect on the early stages in the signal transduction cascade after the binding of growth factors to cell membrane receptors in some types of cells [deGroot, et al., 1991]. Gene expression and the resultant normal growth and differentiation are severely affected. Spaceflight elicits a number of other cellular level responses [Lewis and Hughes-Fulford, in press], and we know that some cell types behave differently in low-g compared to ground, but why this occurs is not understood. A number of organisms and cells flown on sounding rockets and the Space Shuttle have shown changes in biological function compared to ground controls. Some of these changes include a reduction in mRNA and protein synthesis (indication of altered gene expression) in muscle cells, decreased hormone release (secretory process effects) from pituitary cells, and an increase in microtubules (low-g effect on the cytoskeleton) in heart and WI-38 cells. All of these effects and processes are dependent on the transduction of intracellular signals to and from the cell membrane and nucleus.

In salivary gland morphogenesis, cytoplasmic microfilaments and microtubules are involved [Spooner and Wessells, 1972]. There is evidence that the cytoskeleton may be involved in "sensing" gravity at the single cell level. The cytoskeleton functions in signal transduction and the transition of certain cell types from resting to active proliferation. Some of these responses, such as reduced secretion (growth hormone) [Hymer et al., 1985; Grindeland et al., 1990] and blunted T lymphocyte activation [Cogoli 1987; Cogoli and Tschopp, 1985; Cogoli et al., 1984; Cogoli et al., 1987; Bechler et al., 1986] could involve the cytoskeleton; however, this has not been confirmed and the precise mechanisms are as yet unknown.

By investigating molecular mechanisms of SG cell differentiation in the altered gravity vector microgravity bioreactors and in space low-gravity, we hope to advance the understanding of the fundamental biological question of how gravity affects the development and function of individual cells and how cells interact with their environment. The utility of these bioreactors for

ground-based modeling of SG cell growth and differentiation is a preliminary step to conducting research on fundamental biological phenomena in the nonterrestrial environment of space. This dimension opens a vast new area for research into how cells and organisms may have evolved on Earth. Without the unidirectional gravity vector, alternate pathways in signal transduction, gene expression, and differentiation processes appear to become operable in some cell types. Future trends in this research area may be expected to enhance the understanding of the influence of gravity in the development of terrestrial life forms at the cellular level and to provide fundamental information on mechanisms of cellular growth and differentiation.

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