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# Tissue Culture in Microgravity

*by Paul H. Duray, Steven J. Hatfill, and Neal R. Pellis*

**Attempts to simulate normal tissue microenvironments in vitro have been thwarted by the complexity and plasticity of the extracellular matrix, which is important in regulating cytoskeletal and nuclear matrix proteins. Gravity is one of the problems, tending to separate components that should be kept together. For space shuttle experiments, NASA engineers devised a double-walled rotating bioreactor, which is proving to be a useful tissue culture device on earth as well as in space.**

Each tissue in the body is composed of a dynamic assemblage of multiple stationary and migrating cell types that are embedded in a complex macromolecular structure. This extracellular matrix (ECM) consists of polymerized collagens, structural glycoproteins, elastin, glycosaminoglycans, adhesive laminin, and fibronectin, arranged in a complex mesh that is constantly bathed by the fluid of the interstitial tissue space.

The composition of the ECM is variable depending on the type of tissue and its stage of development, and some of the components may undergo a transient change in distribution in response to environmental stimuli or disease states. The cells and composition of the extracellular matrix together with the various cytokines and growth factors found in the interstitial fluid form a series of discrete connected microenvironments which are particular to any given tissue.

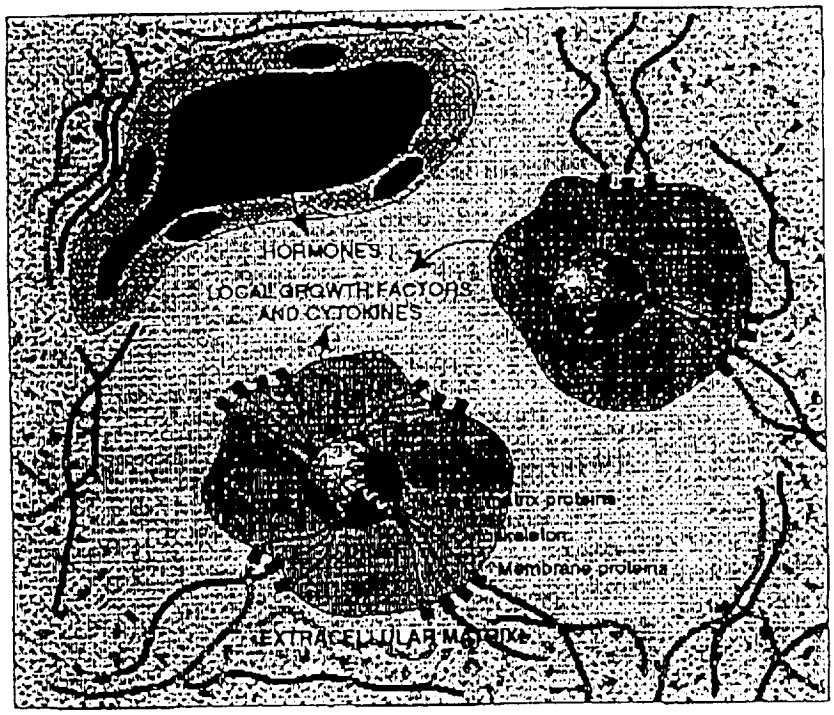
It is well established that cells constantly interact with each other and with their surrounding local microenvironment, and that this communication serves to integrate and coordinate the various gene expression patterns that are crucial for tissue function and homeostasis. Two major signal pathways participate in the cooperative cell communication process.

The first pathway involves the secretion of soluble growth factors and control factors into the microenvironment by the surrounding cells or, in the case of hormones, by cells in distant tissue microenvironments. This signalling process has been recognized for many years, and a number of individual signalling cascades can now be described in intricate biochemical detail.

The second major pathway for cooperative signalling is less well understood. It involves the constant feedback of signals generated by the direct contact of cells with the components of their extracellular matrix. Originally thought to supply only structural support for the cells of a tissue, the ECM is now known to be critical for regulating cell morphology, proliferation, and differentiation, and it is capable of responding to various endogenous and exogenous stimuli.

In vitro studies indicate that cell-generated forces of tension within a tissue can act to organize the ECM into structures that direct the behavior of single cells by influencing cell elongation, alignment, and migration. Such forces also help to create important positional information, which maintains the characteristic three-dimensional histological architecture of a given tissue type.

In addition, the balance between the forces of cellular tension and the viscoelastic resistance of the ECM appears to generate a "solid state" signal between ECM fiber proteins, the cytoskeleton of the cell cytoplasm, and the protein-chromatin scaffold formed by the nuclear matrix. Cooperative signalling interaction between the ECM and the cytoskeleton can effect




An extracellular matrix scaffold not only provides support for a tissue but also transmits important positional and mechanical signals to the cytoskeleton. From the cytoskeleton, these signals are transmitted to nuclear matrix proteins, which act to control the expression of various genes by the cells of a tissue. Between cells, cooperative signaling involves secretion of chemical messengers into the extracellular space. This collection of cells, extracellular matrix, cytokines, and growth factors comprise a microenvironment that is the basis of normal tissue structure and function. Superimposed on the microenvironment is the higher order of control provided by hormones secreted into the bloodstream by cells in distant microenvironments.

changes in the architecture of the nuclear matrix proteins, which in turn can promote the co-localization of transcription factors with the chromosomal regions involved in tissue-specific gene expression.

It has also become apparent that the extracellular matrix indirectly regulates cell behavior by modulating the availability of the soluble growth factors and control factors, which have to diffuse through the ECM mesh to reach their target cells. An increasing number of cytokines, hormones, and growth factors are now known to be able to bind to particular ECM formats. These binding interactions can change the diffusion rate of chemical signals through the microenvironment, activate or sequester various growth factors, and localize or increase the duration of their effects on the surfaces of target cells.

How the extracellular matrix and the microenvironment influence cellular differentiation and tissue function is only beginning to be deciphered, yet several mechanisms have already been implicated in a number of human disease processes, including muscular dystrophy, osteoporosis, glaucoma, liver cirrhosis, and the local invasion and metastasis of cancer cells.

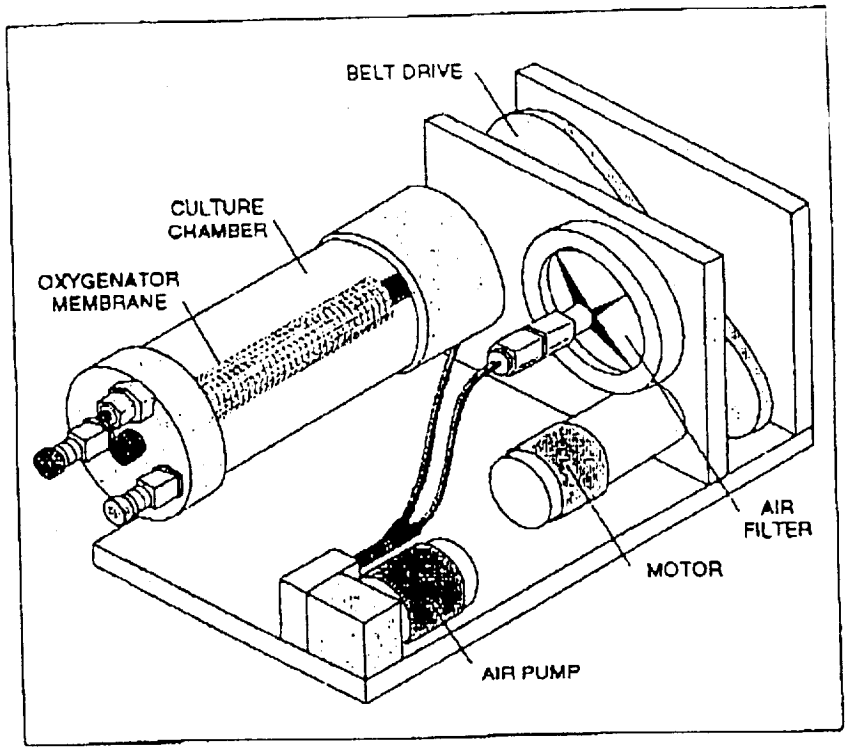
The ability to grow human cells outside the body under static artificial conditions has done much to enhance human health and advance understanding of the physiology and molecular biology of gene expression and regulation in single cells. However, the inherent complexity of three-dimensional extracellular signalling and the remarkable plasticity observed in the composition and structure of the ECM make it difficult to study these interactions using conventional cell culture techniques. More advanced methods are needed for culturing cells in the context of their native three-dimensional cytoarchitecture and tissue microenvironment.

Unfortunately, it has proved extremely difficult to promote the high-density three-dimensional in vitro growth of human tissues that have been removed from the body and deprived of their normal in vivo vascular sources of nutrients and gas exchange. A variety of tissue explants can be maintained for

**PAUL H. DURAY**
received an M.D. from the University of Nebraska College of Medicine. He is board-certified in Anatomical and Clinical Pathology, Dermatopathology, and Cytopathology, and he is presently a specialist pathologist at the National Cancer Institute.

**STEVEN J. HATFILL**
received an M.D. from the University of Rhodesia and a Ph.D. from Rhodes University. A hematological pathologist, Dr. Hatfill is certified in Diving and Submarine Medicine and in Aviation Medicine.

**NEAL R. PELLIS**
was trained as a tumor immunologist and is now Program Director for Biotechnology Cell Science at the NASA Johnson Space Center in Houston.




**Schematic drawing of the rotating wall vessel bioreactor.** An electric motor drives a belt that rotates the culture vessel about its axis. An air pump draws incubator air through a 0.22 µm filter and discharges it through a rotating coupling on the shaft that carries the vessel. The air pump moves about 1 liter of air per minute. An oxygenator membrane is wrapped around the center post.

### BIOREACTORS

In biomedical research, the metabolic activity of cultured cells must be monitored and their environment optimally adjusted to promote both their long-term maintenance and a high cell density. These requirements necessitate well-balanced transport of nutrients to the cultured cells as well as the efficient withdrawal of toxic wastes and inhibitory metabolic byproducts.

A bioreactor is essentially any device that provides the transport system for this process. Based on the principles of fluid dynamics, a number of different bioreactor designs have been developed to optimize oxygen transfer efficiency for a specific cell density and rate of nutrient delivery. Bioreactors range from bench-top laboratory devices to large tanks suitable for commercial biotechnology applications.

a short period of time on a supportive collagen matrix surrounded by culture medium. But this system provides only limited mass transfer of nutrients and wastes through the tissue, and gravity-induced sedimentation prevents complete three-dimensional cell-cell and cell-matrix interactions.

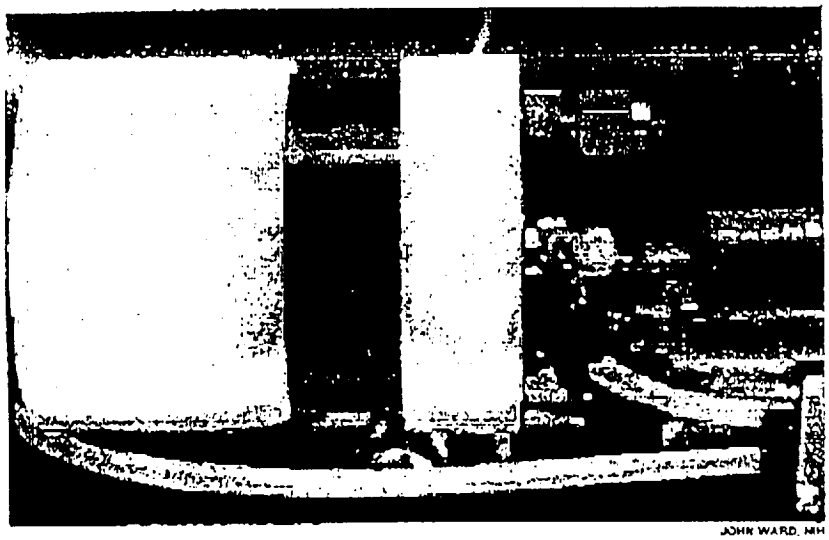
Single-cell co-culture techniques have been developed to overcome these problems; examples are bubble-free oxygenation, porous microcarrier beads, and hollow fiber and fluidized bed bioreactors. These provide excellent mass transfer rates and facilitate high-density cell growth. However, because much of the extracellular matrix and cytoarchitecture in a tissue is laid down during embryogenesis, it is impossible to recreate a normal microenvironment using only collections of well-differentiated cell types.

## NASA Has Developed a Rotating Wall Bioreactor

The fluid-filled rotating wall vessel (RWV) bioreactor is a recently developed cell culture device that is able to successfully integrate cell-cell and cell-matrix co-localization and three-dimensional interaction with excellent low-shear mass transfer of nutrients and wastes, without sacrificing one parameter for the other. Designed by Ray Schwarz and assistant engineers in conjunction with the Johnson Manned Spaceflight Center, the RWV bioreactor consists of a cylindrical growth chamber that contains an inner co-rotating cylinder with a gas exchange membrane.

Cells and liquid culture media are placed in the space between the inner and outer cylinders, and the assembled device is rotated about its longitudinal axis. Because of viscous coupling, the liquid inside the vessel accelerates until the entire fluid mass is rotating at the same angular rate as the outside wall. Microcarrier beads and cells in this environment obey simple kinematics and are uniformly suspended in the fluid at a given rotational speed.

The suspended cells rotate as a solid body with minimal disruptive shear, and the cells maintain their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The RWV bioreactor culture chamber rotating at 35 rpm, showing the free-fall behavior of explanted blocks of human tonsillar tissue. The tissue explants retained a normal histological cytoarchitecture after 10 days of continuous culture under simulated microgravity conditions.

relative positions for long periods, allowing them to touch one another or to construct bridges between the microcarrier beads. In addition, chamber rotation subjects the cells to a constantly changing angular gravity vector. Constant randomization of the normal gravity vector subjects the cells to a state of simulated free fall, similar in some respects to a microgravity environment and akin to the free fall experienced for much shorter periods by aircraft in parabolic flight.

The fluid dynamic operating principles of the RWV culture system thus encompass solid body rotation about a horizontal axis with some degree of three-dimensional spatial freedom, oxygenation without turbulence, high mass transfer rates, low fluid shear forces, and the co-localization of particles that have different sedimentation rates.

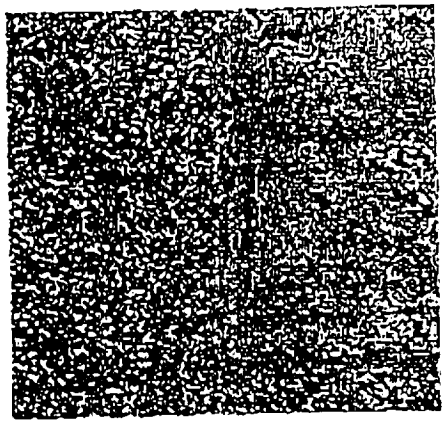
The RWV bioreactor was originally intended to protect delicate cell cultures from the high shear forces generated during the launch and landing of the space shuttle. When the device was tried for cell-line suspension cultures on the ground, cells were seen to aggregate and form larger structures resembling tissues. This observation offered the exciting possibility that the bioreactor might be used to study the interactions of multiple cell types and their association with proliferation and cellular differentiation during the early steps of tissue formation.

## Tissue Equivalents Can Be Constructed

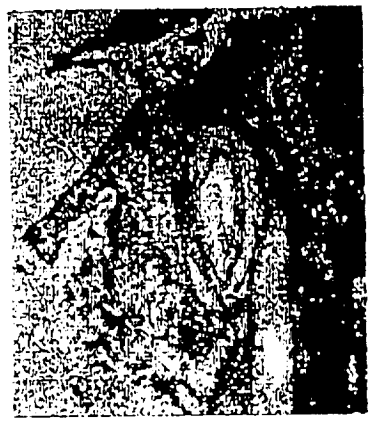
The rotating wall vessel bioreactor has already been used to develop a number of three-dimensional cell models that can mimic in vivo responses particular to certain types of tissue. At the University of Pennsylvania, Portonovo Ayyaswamy and colleagues have used the RWV bioreactor to co-culture rat bone marrow stromal cells together with suspended microcarrier beads. They observed the formation of complex 600 µm structures bound together by a collagen-rich ECM, with calcium phosphate deposited within the newly secreted matrix between the beads.

The RWV bioreactor has been recently used at the University of Wisconsin to co-culture rat adrenal chromaffin cells together with a microvascular endothelial cell line. Self-forming "organoids" were observed after 20 days in culture. They contained normal-appearing adrenal cells surrounded by an extracellular matrix composed of fibronectin and type IV collagen, and they continued to grow until they reached sizes similar to a normal rat adrenal gland.

## Human Tissues Cultured in the RWV Bioreactor

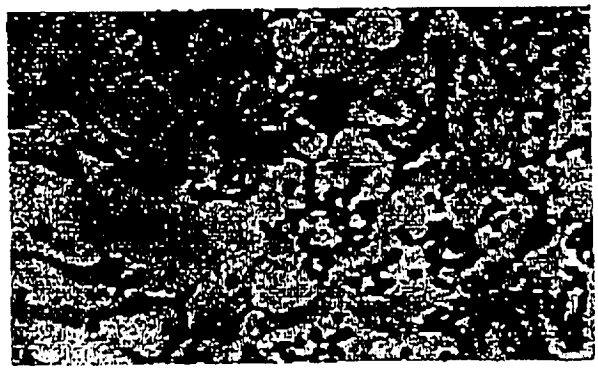


**Accessory salivary gland tissue** cultured for 14 days shows intact endothelium, myoepithelial cells in the minor salivary gland lobules, and lymphocytes in the tissue stroma. H&E stain. Original magnification X 200.

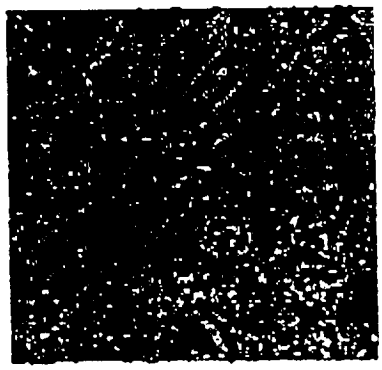


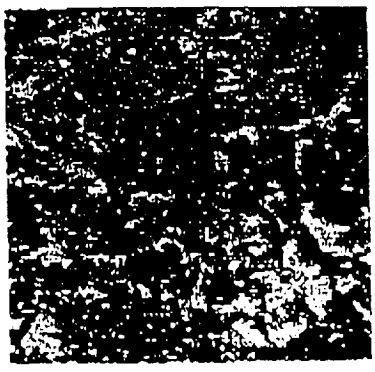
**Full-thickness human skin explants** grown for 14 days in the RWV bioreactor. The *left panel* shows continued preservation of an intact basal cell layer over subcutaneous tissue. Higher-power views revealed the presence of melanocytes in the sections. The *right panel* shows maintenance of intact hair follicles. H&E stain.

**Human liver tissue** obtained four hours post mortem and cultured as 0.5 mm explants for five days. The hepatic cords are intact, and the brown staining is indicative of bilirubin production by some of the hepatocytes. H&E stain. Original magnification X 600.



**Bone marrow cells** obtained as a by-product of total hip replacement and grown for seven days on an artificial collagen matrix. Lymphocytes, myeloid precursors, erythroblasts, and stromal cells can be identified by immunohistochemical staining. H&E stain. Original magnification X 800.





**Lung carcinomas.** *Left panel,* Section of an adenocarcinoma biopsy taken after seven days in culture shows viable neoplastic cells with invasion into the surrounding tissue. H&E stain. Original magnification X 800.

*Right panel,* Section of a lung biopsy taken after 14 days in culture shows virtually complete replacement of normal tissue with squamous carcinoma. H&E stain. Original magnification X 100.

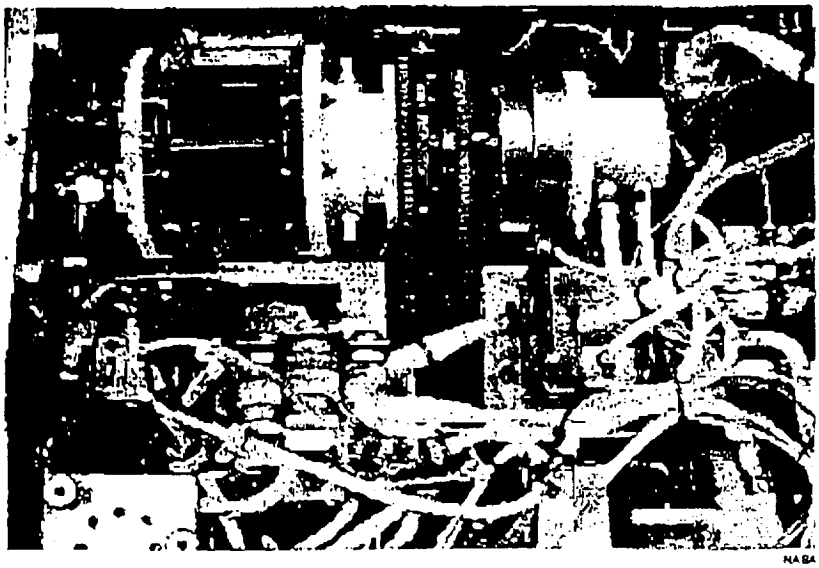
Other investigators have shown that human carcinoma cells in RWV culture can aggregate into larger three-dimensional multicellular aggregates exhibiting differentiated glandular structures that are capable of secreting mucin.

Initial experiments with single-cell suspensions and "organoid" formation in the RWV bioreactor suggested that this culture system might be a useful tool for constructing tissue equivalents, which are in vitro models of tissue made by co-localizing its major cell types within a single culture flask. At the University of North Texas Health Science Center, S. Dan Dimitrijevich and co-workers have used the RWV bioreactor to generate human skin equivalents from suspension cultures composed of isolated fibroblasts, keratinocytes, and stratified squamous epithelial cells. A number of laboratories are exploring the ability of the RWV bioreactor to create several types of important tissue equivalents.

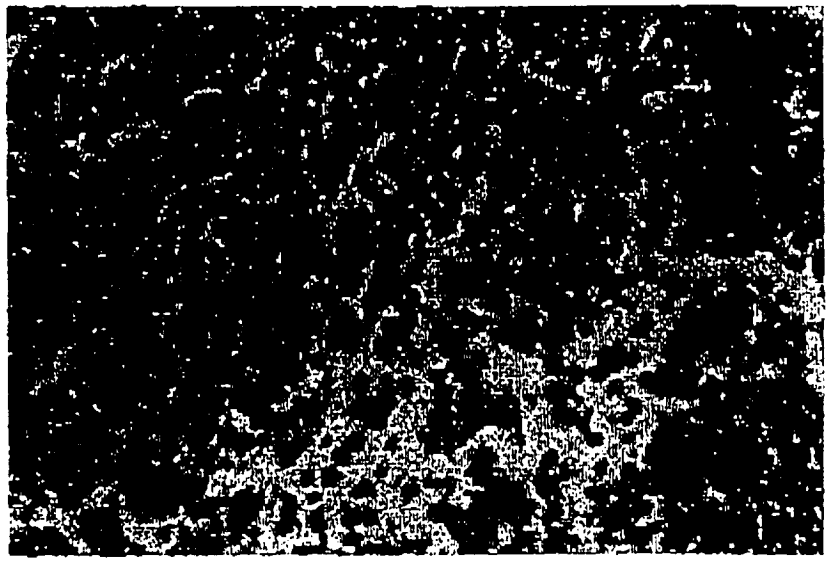
Cartilage and endochondral bone formation is an example of a developmental process in which the extracellular matrix and microenvironment play critical roles. Understanding the mechanisms involved in the formation of these tissues would have important clinical significance for a variety of human disease processes. Initial experiments at the NASA Johnson Space Center demonstrated that embryonic limb mesenchymal cells could form aggregates containing more differentiated chondrocytes that responded to the RWV culture conditions by secreting a variety of ECM components.

Spurred by the possibility of using matrix-secreting RWV cultured chondrocytes as clinical implants, Lisa Freed at the Massachusetts Institute of Technology and researchers at Boston University and the Georgia Institute of Technology have explored the use of the RWV bioreactor to construct cartilage tissue equivalents. Bovine chondrocytes were cultured using a polyglycolic acid polymer scaffold and various cytokine cocktails to form cartilage tissue constructs.

On earth, these constructs were found to form sheets 2 mm thick, with normal-appearing lacunae and cartilage-specific ECM proteins comparable to normal mammalian cartilage. With the proper culture media, the constructs were shown to be able to undergo mineralization, similar to normal endochondral bone formation in the body. Duplicate RWV experiments that were flown for four continuous months on the Mir space station




**Flight-ready hardware** designed by NASA for RWV bioreactor experiments on the space shuttle and Mir space station. Culture conditions within the RWV chamber are continuously monitored and optimized during space flight by microprocessor control. In this version, the inner wall of the chamber rotates at a slightly different angular rate than the outside wall to promote mixing of nutrients and dissolved gases in the culture medium during exposure to the microgravity environment of space.



**Human tonsil tissue** grown for 10 days in the RWV bioreactor. Morphologically viable lymphocytes are located within an intact follicle germinal center. H&E stain. Original magnification X 600.

have demonstrated important differences between cartilage tissue equivalents formed on earth and in space. Such experiments will lead to a better understanding of the effects of long-duration space flight and may provide new strategies for investigating bone repair, osteoporosis, and osteoarthritis.

## Tissue Explants Can Be Maintained for Long Periods

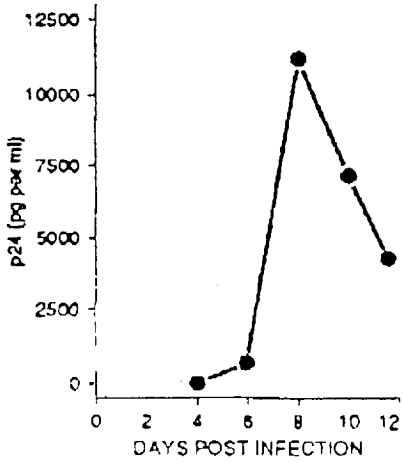
The ability of the RWV bioreactor to model certain aspects of tissue formation, such as the secretion of extracellular matrix components and construction of tissue equivalents, during the co-culture of multiple cell types has prompted investigators to examine the possibility that RWV culture might be able to maintain the actual native microenvironments and cytoarchitecture of explanted human tissues. Experiments by Joshua Zimmerberg and Leonid Margolis at the National Institutes of Health have shown that the RWV bioreactor can maintain the structure of human lymphoid tissue explants.

Blocks of tonsil tissue subjected to RWV culture have been shown to be able to maintain a normal CD4 to CD8 T cell ratio and a histologically normal cytoarchitecture for up to three weeks in vitro. The RWV explants are capable of generating a functional secondary immune response to selected antigens and can support a productive infection by HIV-1 without the addition of exogenous cytokines to the culture medium. During HIV infection, the lymphoid tissue explants demonstrated progressive loss of CD4 cells and the development of a disorganized cytoarchitecture similar to that observed in AIDS patients. Addition of the antiviral agent AZT to the RWV culture medium inhibited HIV replication within the cultured blocks of lymphoid tissue.

The RWV bioreactor is also being used to test the effectiveness of other antiviral agents in the context of a native tissue microenvironment. Mike Bray and his coworkers at the U.S. Army Medical Research Institute of Infectious Diseases at Fort Detrick are currently using RWV cultured explants of human liver, spleen, and lymph nodes in an effort to find new compounds that could be used to treat patients infected with the lethal Ebola virus.

In other experiments, the NASA bioreactor has been able to preserve the normal three-dimensional structure of explanted human prostate tissue in culture. After an

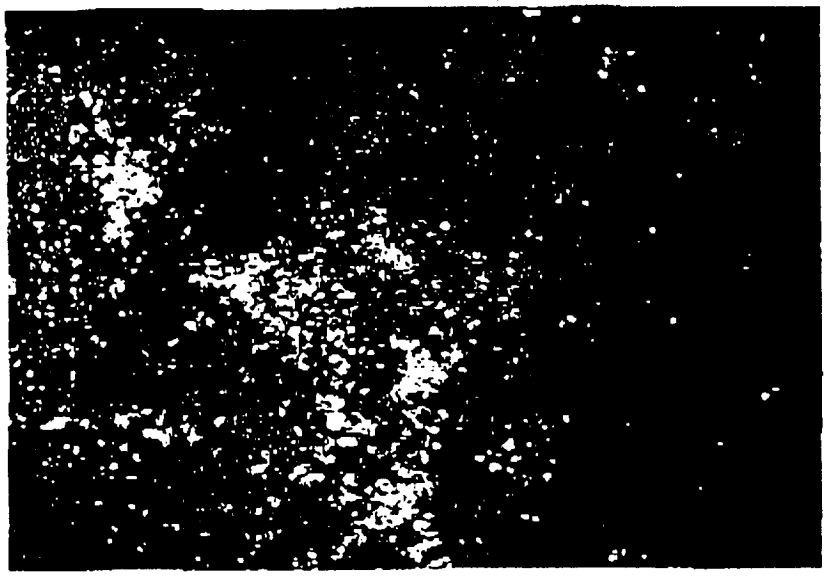
**Productive infection by HIV-1** is supported by human lymphoid tissue cultured in the RWV bioreactor without the addition of exogenous cytokines or growth factors. The graph shows the accumulation of HIV-1 p24 protein in the culture medium of an RWV bioreactor that contained blocks of human tonsil tissue infected with HIV-1.



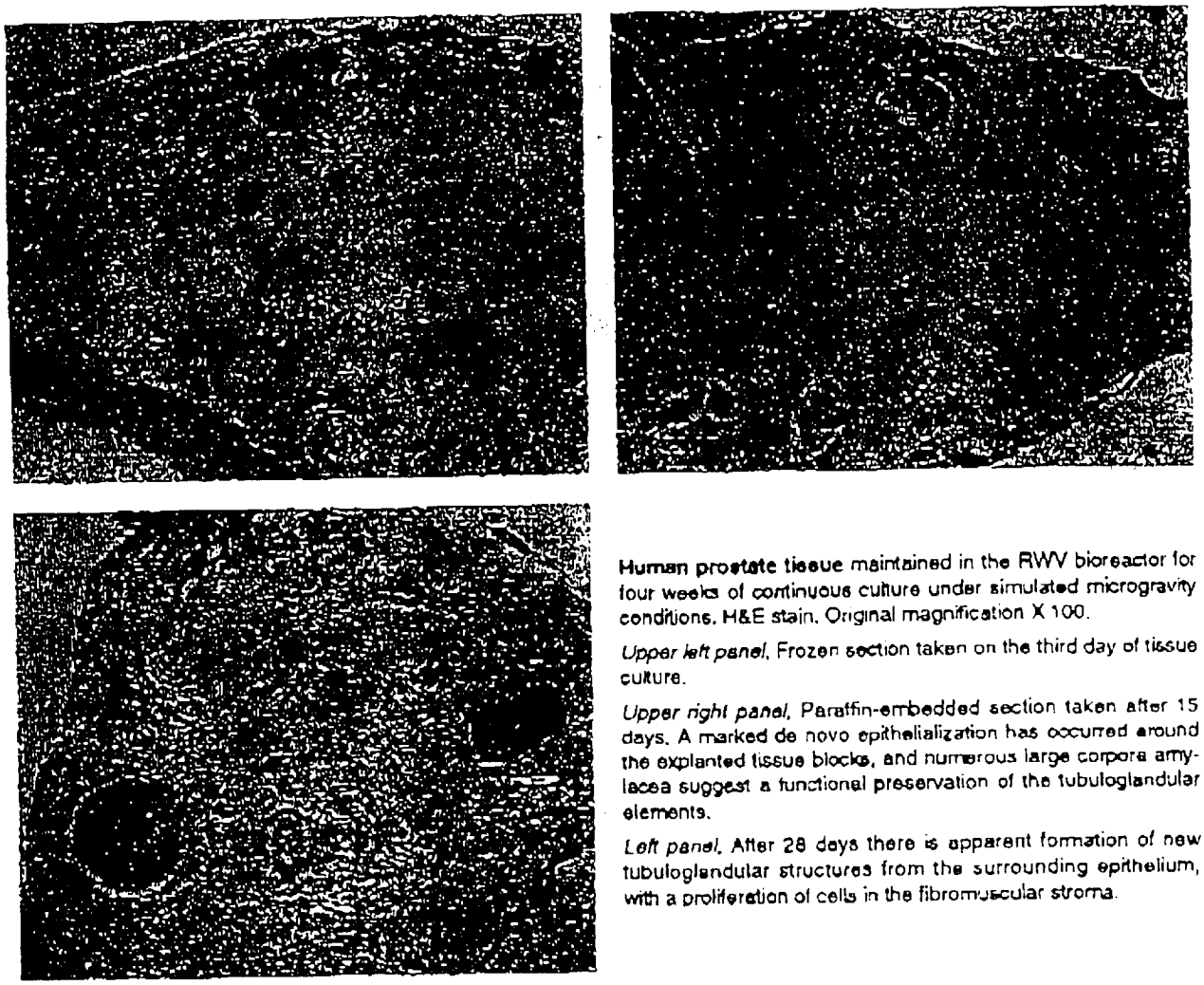

The human P4 pathogenic virus Ebola-Zaire replicating in human tonsillar tissue cultured for six days post infection in the RWV bioreactor. The infectious Ebola virus particles are the elongated electron-dense structures in the center of the transmission electron micrograph.

initial period of reorganization, explanted blocks of prostate tissue have been shown to maintain both tubuloglandular and fibromuscular stromal elements during six weeks of continuous RWV culture. When added to the culture medium of the bioreactor, malignant prostate epithelial cells can attach to and invade the normal fibromuscular stroma of the tissue. This provides a useful model for the study of host-tumor cell interactions in the human prostate.

The development of a variety of human epithelial cancers is thought to follow an ordered progression from metaplasia to dysplasia and, later, to carcinoma in situ. Bruce Johnson and his associates at the Medicine Branch of the National Cancer Institute are using the NASA bioreactor to study the metaplastic phenotype in human bronchial epithelium. Using microdissection and reverse transcription PCR, these investigators are trying to isolate the genetic changes responsible for the preneoplastic phenotype and to discern the possible effect of retinoids in reversing the process.



Human lymphocytes constantly circulate from the peripheral blood into the complex microenvironments of lymphoid tissues and back into the bloodstream. This picture, obtained by confocal microscopy, shows the ability of the RWV bioreactor to model lymphocyte trafficking. Fluorescently labeled human lymphocytes were placed into the culture medium along with co-cultured explanted blocks of human spleen. After five days of continuous culture, some of the labeled lymphocytes have migrated from the culture medium to take up residence in the splenic microenvironment.



**Human prostate tissue** maintained in the RWV bioreactor for four weeks of continuous culture under simulated microgravity conditions. H&E stain. Original magnification X 100.

*Upper left panel,* Frozen section taken on the third day of tissue culture.

*Upper right panel,* Paraffin-embedded section taken after 15 days. A marked de novo epithelialization has occurred around the explanted tissue blocks, and numerous large corpora amylacea suggest a functional preservation of the tubuloglandular elements.

*Left panel,* After 28 days there is apparent formation of new tubuloglandular structures from the surrounding epithelium, with a proliferation of cells in the fibromuscular stroma.

## Tissue Engineering Is the Most Recent Application

Tissue engineering is the growth and modification of human tissues within the context of their normal three-dimensional microenvironment, with the aim of implanting such tissues back into the body. Recent research suggests that the unique environment provided by the RWV bioreactor facilitates the growth and continued function of a number of different tissue explants, and it is conceivable that genetic modification of such explants might eventually be used to treat children born with enzyme deficiencies.

Gene therapy experiments to date have focused on the use of bone marrow stem cells to replace defective genes with normal ones that can be expressed in the hematopoietic compartment. Unfortunately, hematopoietic stem cells are difficult to isolate, and transfection of these cells with normal genes is an inefficient process. Further advances will likely require a different type of target cell.

In this respect, the hepatocyte seems to be an ideal candidate. The possibility of forming stable long-term liver tissue equivalents from human hepatocytes is currently being studied by Boris Yoffe and his colleagues at Baylor College of Medicine. Partially purified hepatocytes and biliary epithelial cells are successfully co-cultured as self-forming aggregates arranged on a

Normal human lung tissue grown in the RWV bioreactor for 10 days shows preservation of alveolar sac walls. H&E stain. Original magnification X 400.

polymer scaffold and have persisted for up to 60 days under RWV culture conditions. These three-dimensional liver tissue equivalents develop proliferating hepatic cords and new bile ducts, as well as new vascular endothelial structures.

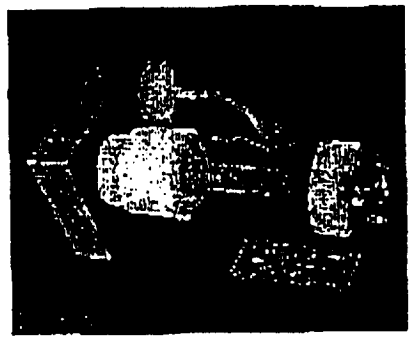
It is conceivable that further research may eventually allow these liver tissue equivalents to be genetically engineered and subjected to a process of initial vascularization in the RWV bioreactor before they are transplanted back into the body to repair certain inborn errors of metabolism in human patients.

Promotion of spinoff applications of NASA technology has always been a part of the American space program, and NASA scientists have made significant contributions to telemedicine, physiological monitoring, cell and radiation biology, biosensors, and laboratory instrumentation and automation.

The unique cell culture environment provided by the RWV bioreactor has prompted NASA to license the technology for commercial applications. Complete self-contained versions of the bioreactor are being manufactured by Synthecon, Inc., a Houston biotechnology company. These bioreactors will enable even small laboratories to conduct tissue research under simulated microgravity conditions. Also, VivoRx of Santa Monica is using RWV technology as a strategy to grow large numbers of insulin-secreting human pancreatic islet cells for use in clinical trials.

The number and variety of ongoing research and application programs make it likely that NASA's rotating wall vessel bioreactor will continue to generate useful information about the structure and function of normal tissues and the pathogenesis of a wide variety of important human diseases.

The Synthecon self-contained RWV bioreactor is designed to enable smaller laboratories to culture cells and tissue under simulated microgravity conditions. The apparatus is designed to be placed inside a humidified $CO_2$ incubator.



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