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invited review

Optimized suspension culture: the rotating-wall vessel

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Hammond, T. G., and J. M. Hammond. Optimized suspension culture: the rotating-wall vessel. *Am J Physiol Renal Physiol* 281: F12–F25, 2001.—Suspension culture remains a popular modality, which manipulates mechanical culture conditions to maintain the specialized features of cultured cells. The rotating-wall vessel is a suspension culture vessel optimized to produce laminar flow and minimize the mechanical stresses on cell aggregates in culture. This review summarizes the engineering principles, which allow optimal suspension culture conditions to be established, and the boundary conditions, which limit this process. We suggest that to minimize mechanical damage and optimize differentiation of cultured cells, suspension culture should be performed in a solid-body rotation Couette-flow, zero-headspace culture vessel such as the rotating-wall vessel. This provides fluid dynamic operating principles characterized by *1*) solid body rotation about a horizontal axis, characterized by colocalization of cells and aggregates of different sedimentation rates, optimally reduced fluid shear and turbulence, and three-dimensional spatial freedom; and *2*) oxygenation by diffusion. Optimization of suspension culture is achieved by applying three tradeoffs. First, terminal velocity should be minimized by choosing microcarrier beads and culture media as close in density as possible. Next, rotation in the rotating-wall vessel induces both Coriolis and centrifugal forces, directly dependent on terminal velocity and minimized as terminal velocity is minimized. Last, mass transport of nutrients to a cell in suspension culture depends on both terminal velocity and diffusion of nutrients. In the transduction of mechanical culture conditions into cellular effects, several lines of evidence support a role for multiple molecular mechanisms. These include effects of shear stress, changes in cell cycle and cell death pathways, and upstream regulation of secondary messengers such as protein kinase C. The discipline of suspension culture needs a systematic analysis of the relationship between mechanical culture conditions and biological effects, emphasizing cellular processes important for the industrial production of biological pharmaceuticals and devices.

laminar flow; bioproducts; microcarriers; shear; gene expression

SUSPENSION CULTURE

MOST IF NOT ALL DIFFERENTIATED cells derived from diverse tissue sources lose their specialized features and dedifferentiate when grown under traditional two-dimensional cell culture conditions (24, 25, 68). Suspension culture is the most popular means of preventing this problem and maintaining specialized features of cells. The rich and diverse array of available culture vessels for suspension culture makes selection of specific culture devices bewildering. There is a great deal of information available on the optimization of suspension culture by utilizing the engineering principles of

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fluid physics to minimize shear and turbulence (20, 24, 45, 47, 60, 61). However, for most biologists, translating this technical information into practical culture conditions is complex and counterintuitive.

Several recent reviews have summarized the use of suspension culture (2, 26), scale-up of suspension culture for industrial use (44), application to anchorage-dependent cells by the use of microcarriers (9, 20, 45), and automation of roller-bottle forms of suspension culture (37). Some earlier reviews have collected the body of data on the use of selected culture devices such as the rotating-wall vessel (57, 68). This review has two purposes not addressed by any of these other documents. The first is to summarize the engineering principles of suspension culture in a manner that will facilitate the choice of application-specific hardware, elucidate the limits of the technique, and aid in establishing optimal suspension culture conditions. Second, this review brings together available data on the mechanisms by which suspension culture mediates such dramatic changes in the properties of diverse types of cultured cells.

PHYSIOLOGICAL IMPORTANCE

Although an old approach, suspension culture only increases in importance over time, as the demand for cell cultures maintaining differentiated features increases for both academic and industrial applications (24, 33, 68). Suspension culture continues to be useful for production of many bioproducts, from antibodies to hormones (31, 33, 36). The availability of cell culture models, which are so easily modulated and studied, with an intact tissue-specific repertoire of signal transduction and metabolic pathways would represent a dramatic biotechnology advancement. Hence, despite present limitations on the ability to access the basal cell membrane on beads for studies of transcellular transport, differentiated suspension culture cells have broad practical application. Engineering optimization of suspension culture was largely undertaken by National Aeronautics and Space Administration (NASA) engineers to model culture conditions in spaceflight (57, 72, 73) but may find its greatest utility in the carryover to ground-based applications (ftp://ftp.hq.nasa.gov/pub/pao/pressrel/2000/00–143.txt).

TIMELINESS

The maturation of three major initiatives that utilize suspension culture techniques makes a review of the principles of suspension culture timely. First, an understanding of the physics and engineering principles is definitive but not freely available or reviewed in Medline-indexed journals (20, 22, 23, 36, 51, 52, 57, 72, 73). Many investigators contemplating suspension culture are likely to find a simple, direct summary extremely helpful.

Second, new methods to monitor and quantitate the effects of suspension culture on cells have become readily available (4, 16, 42, 43). The human genome project, and the associated production of huge libraries of expressed sequence tags, provided momentum for the development of new methods for assaying large numbers of genes simultaneously (4, 16, 42, 43). Gene (expression) array analysis has provided a rapid, inexpensive, but sophisticated method to meet these needs. Gene array has provided a revolution in the search for new drugs by monitoring a drug's effects on thousands of genes, making it possible to predict the usefulness of cell culture systems as model systems for both in vitro and in vivo toxicity testing (4, 16, 42, 43).

Finally, long used to produce antibodies and other products for clinical use, suspension cultures are now translating directly into clinical practice (33, 38, 56). Pancreatic islets are presently maintained alive, differentiated, and with regulated insulin secretion in rotating-wall vessel suspension culture before experimental and human transplantation (53–56, 59, 70). Suspension culture is used for industrial fermentation reactions (33), antibiotic production (38), interferon manufacture, and other bioproducts from hormones to hybridomas (35, 60; ftp://ftp.hq.nasa.gov/pub/pao/pressrel/2000/00–143.txt).

OPTIMIZING CELL CULTURE IN SUSPENSION: THE ROTATING-WALL VESSEL

Engineering, Biology, and NASA

When biotechnology problems require an engineering solution, NASA's massive repository of engineering expertise has on occasion been applied to biological problems. Probably the best known and most fully developed example of this collaboration is the NASA/Baylor/DeBakey axial flow ventricular assist device or "artificial heart" (15, 31). After the heart failure death of a NASA engineer compromised by a prolonged wait for a donor transplant heart, NASA engineers played an integral role in the development of a low-shear, high-capacity blood pump. The NASA engineers initially donated thousands of man-hours and then continued with the support of a variety of Federal and private funding sources (15). NASA engineering was pivotal to the development of a pump combining the capacity to move the necessarily quite large blood volumes with preservation of the integrity of red blood cells and other blood elements (15, 31).

Similarly, NASA's Biotechnology Group, based at the Johnson Space Center in Houston, TX, has modeled the problem of optimizing mechanical culture conditions in suspension culture by minimizing shear and turbulence (22, 23, 51, 52, 57, 67, 72, 73). This led to a radical new (but simple) design for suspension culture vessels embodied in the rotating-wall vessel, a horizontally rotating cylindrical culture vessel with a coaxial tubular oxygenator (Fig. 1). These vessels have characteristic features that determine their utility. First, fluid flow is near solid body or laminar at most operating conditions. This avoids the large shear stresses associated with turbulent flow and allows introduction of controlled and nearly homogenous shear fields. If the inner cylinder and outer cylinders of the rotating-wall vessel rotate at the same angular velocity (rpm), then

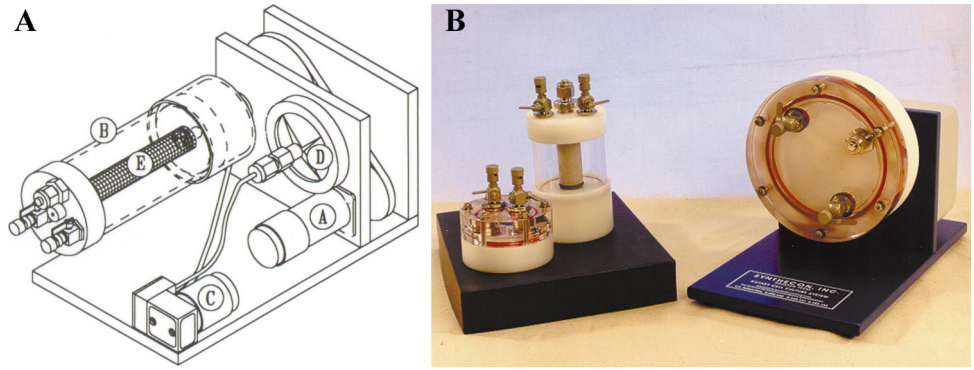

Fig. 1. Rotating-wall vessels. *Left*: schematic of the rotating-wall vessel. A 24-V direct-current motor (*A*) drives a belt that rotates the cylindrical culture vessel (*B*) along its horizontal axis. An air pump (*C*) draws incubator air through a 0.22-μm filter (*D*) and discharges it through a rotating coupling on the shaft that carriers the vessel. The oxygenator (*E*) is wrapped around the center post. *A* is reproduced from Ref. 22; © Wiley-Liss, Inc.; reprinted by permission. *Right*: photograph of the rotating-wall vessel in operation. Selected vessel volumes and designs are shown.

the laminar flow fluid velocity gradient (radially) is minimized. This minimizes the actual laminar flow and totally eliminates the associated shear stress. The shear stress at any point in the fluid is the product of the fluid velocity gradient and viscosity. Second, the culture medium is gently mixed by rotation, avoiding the necessity for stirring vanes. The mixing is the result of a secondary flow pattern induced by particle sedimentation through the fluid media or by laminar flows established when differential rotation rates are chosen for the vessel components. Stirring vanes damage cells by both local turbulence at their surface and the high flow rates created between the vessel walls and the vanes. The vanes or any other "in-vessel" mixing apparatus invariably causes inhomogeneity in the shear field, often of large magnitudes, which confounds the interpretation of culture results. Third, there is no headspace. In roller bottles, due to incomplete filling of the vessel, the air in the headspace creates turbulence and secondary bubble formation in the culture medium, which are both potent sources of extra shear and turbulence. Fourth, anchorage-dependent cell types can be grown on microcarriers, just as in other suspension devices (23, 64). Last, the coaxial oxygenator and the outer wall may rotate at the same slow (10–60 rpm) rate, avoiding and/or minimizing shear from differential rotation (see below) or that produced by a vane passing the stationary wall of a spinner flask.

The rotating-wall vessel largely solves the challenges of suspension culture: to suspend cells and microcarriers without inducing turbulence, or large degrees of shear, while providing adequate nutrition and oxygenation. Earlier reviews of the most efficient bioreactor design and agitation regimens concluded that conditions of turbulent flow existed in all stirred tank bioreactors (7, 8, 12–14). The ability of cells to proliferate in horizontally rotating vessels had been demonstrated (3) but never optimized or combined with solutions of the oxygenation and nutrition problems. Oxygenation by bubbling or sparging is another potent source of mechanical cell impact and damage (6, 58). This problem is overcome by the continuous delivery of large volumes of oxygen along an axial oxygenator, using only dissolved gases without bubbles for exchange (22, 51, 72). Last, intermittent batch feeding or perfusion can provide nutrition (22, 51).

In summary, the rotating-wall vessel provides fluid dynamic operating principles characterized by *1*) solid body rotation about a horizontal axis that is characterized by colocalization of cells and aggregates of different sedimentation rates, optimally reduced fluid shear and turbulence, and three-dimensional spatial freedom; and *2*) oxygenation by diffusion, excluding undissolved gases from the bioreactor (22, 51, 72, 73).

PRINCIPLES OF OPTIMIZED SUSPENSION CULTURE IN THE ROTATING-WALL VESSEL: TRADEOFFS

The rotating-wall vessel has simple features designed to provide continuous sedimentation of particles through culture medium, which is rotating essentially as a solid mass with minimal induced cellular shear and turbulence. Three tradeoffs inherent in the operation of the rotating-wall vessel are key to both defining the limits of the technology and optimizing the mechanical cell culture conditions (19, 20, 34–36, 60, 72) (Table 1). The first tradeoff results from the determinants of terminal velocity in the vessel. The second tradeoff results from a balance of Coriolis and centrifugal forces induced by rotation of the gravitational vector. Third, mass transfer is effected by both terminal velocity and the diffusion properties of the culture medium. To optimize suspension culture, these tradeoffs need to be expressed in simple mathematical relationships, quantitated, and controlled.

Tradeoff I: Determinants of Terminal Velocity and Shear in the Rotating-Wall Vessel

Terminal velocity. A cell or cell aggregate in suspension culture in the rotating-wall vessel accelerates through the fluid until it reaches a terminal velocity

Table 1. *Tradeoffs in the rotating-wall vessel*

Tradeoff	Nature	Optimization
I	Terminal velocity determinants	Match particle and culture medium density as closely as possible Reduce gravity in spaceflight experiments
II	Coriolis and centrifugal forces	Adjust rpm for minimum total Coriolis- and centrifugal-induced spiral excursion Minimize terminal velocity (as in *tradeoff I*)
III	Mass transfer	Minimize viscosity of culture medium Provide concentration gradient for nutrients

(V_s), at which the pull of gravity is balanced by equal and opposite hydrodynamic forces including shear, centrifugal, and Coriolis forces. The shear on a particle is proportional to the terminal velocity of the cell aggregate, which is in turn determined by *Eq. 1* (22, 51, 67, 72)

$$V_s = [2gr^2(\rho_\rho - \rho_f)]/(9\mu\rho_f) \quad (1)$$

where V_s is the terminal velocity, g is gravity, r is the radius of the particle, ρ_ρ is the density of the culture particles, ρ_f is the density of culture medium (fluid), $\rho_\rho - \rho_f$ is the difference in density between culture particles and medium, and μ is the viscosity of cell culture medium.

Hence the major direct determinants of the terminal velocity of a cell aggregate in suspension culture are gravity and the radius of the particle squared (see Table 2). There are direct effects of cell and fluid density and inverse effects of viscosity and density of the culture medium. All these parameters are easily modified and optimized.

Shear stress. The maximum shear stress (τ_{max}) is a function of the terminal velocity as shown by the following (1, 20, 72)

$$\tau_{max} = \frac{3\mu V_s}{2r} \quad (2)$$

where V_s, r, and μ are defined as in *Eq. 1*.

Equation 1 applies to creeping flow around a solid sphere (2), which adequately describes the movement of a particle inside the rotating-wall vessel. This is true when the Reynolds number (which is a dimensionless quantity associated with the smoothness of flow of a fluid, roughly the ratio of the flow speed to the viscosity of the fluid), [$Re = 2R_{\rho_f}V_t/\mu$], is <0.1 (3), which is the case for all commonly employed Cytodex or glass beads with radii <275 μm, where the parameters are defined as above.

Table 2. *Determinants of terminal velocity and shear stress in the rotating-wall vessel*

Parameter	Description	Relationship	Power
	Terminal velocity		
g	Gravity	Direct	1
r	Radius of particles	Direct square	2
$\rho_\rho - \rho_f$	Difference in density between culture particles and medium	Direct	1
μ	Viscosity of cell culture medium	Inverse	−1
ρ_f	Density of culture medium	Inverse	−1
	Shear stress		
μ	Viscosity of cell culture medium	Direct	1
V_s	Terminal velocity	Direct	1
r	Particle radius	Inverse	−1

Gao et al. (14), in their model of bead motion in the rotating-wall vessel, defined a relative velocity (V_{rel}) between the bead and the fluid and used *Eq. 2* to calculate shear stress at the bead surface, with V_{rel} in place of V_s. They show the periodic nature of the relative velocity and hence of shear stress, but, because the oscillations are slight, these values may be assumed constant for a given set of conditions. The authors define a system in terms of the following physical parameters: fluid viscosity (μ), density difference ($\Delta\rho$) between particle and fluid, microcarrier bead radius (a), vessel rotational speed (ω), and initial particle position (r_0) (see Fig. 3). They found that the maximum shear stress increases linearly with particle radius and density difference. Maximum shear stress is constant, however, with respect to fluid viscosity and vessel speed. Note that the increase in particle velocity through the fluid is not appreciably increased due to the centrifugal or Coriolis effects because these are small compared with the gravitational effects. The authors suggest that the opposing effect of viscosity on relative velocity cancels its effect on shear stress. Although τ_{max} is not dependent on ω or μ, another important parameter is the time until wall impact, which varies inversely with ω and increases linearly with μ. Thus there will be more wall impacts at higher vessel speeds and lower fluid viscosities. From this analysis, we can conclude that the major determinants of shear on a cell aggregate in the rotating-wall vessel are density difference and particle radius.

These simple mathematical relationships make several testable predictions about suspension culture (50, 51, 72, 73). First, changes in gravity, such as placing a suspension culture in a centrifuge or growing cells in the microgravity of space, should have dramatic effects on terminal velocity and hence on shear. Second, as aggregates grow in size during suspension culture, there is a secondary, geometrically related increase in shear. This predicts that without changing gravity, suspension cultures are gravity limited: as the size of the aggregates increases, the shear increases exponentially, leading to cellular damage and aggregate disruption.

The first tradeoff in the rotating-wall vessel is that gravity must be balanced by equal and opposite me-

chanical forces. These forces produce shear, but this can be minimized by matching the densities of the cell aggregates and culture medium as closely as possible or reducing gravity as occurs during spaceflight studies.

Practical implications. The physics of the rotating-wall vessel determines that the shear stress is fixed by gravity, and this has vital practical implications. This relationship means that every time the rotating-wall vessel containing the same cells and culture medium is turned on, it delivers exactly the same terminal velocity and consequent shear stress to similar-sized particles, no matter how much the rotation speed may vary. This is essentially a foolproof system. Contrast this with the physics of a spinner flask, in which minor changes in speed create dramatic changes in shear stress (22, 72) that are further confounding due to the dramatic time and spatial inhomogeneity of the shear field. Given the tiny dynamic range of shear stress in which many cultured cell types flourish (22, 57) before being irreversibly damaged by the ensuing physical forces, minor increases in spinner vane speed often have catastrophic consequences for the structural integrity of cell cultures.

Tradeoff II: Coriolis Spiraling Due to Radial and Tangential Components of the Gravity Vector

The resolution of the gravity vector acting on a particle in the rotating-wall vessel into radial and tangential components is of great practical importance as it explains and quantitates the slow spiraling of particles in the vessel and leads to an explanation of the limitations of the vessel's efficacy (and hence suspension culture efficacy) (20, 36, 46, 50, 51, 67, 72, 73).

The circumference of the circular particle motion is only determined by the sedimentation rate and vessel rotation rate. Secondary, low-order Coriolis motions result only from the radial velocity components, which themselves are determined by gravity sedimentation and centrifugation. These are negligible in practice but contribute to a slow lagging of the particles behind the vessel's angular position.

As the fluid rotates in a rotating-wall vessel, the gravitational vector acting on a particle can be resolved into radial and tangential components (20, 36, 50, 72) (Fig. 2*A*). That is, the diameter of the spirals of particles cultured in suspension in the rotating-wall vessel is determined by the terminal velocity (see *Eq. 1* and Table 2), the angular velocity of the vessel, gravity, and the radial particle velocity. These relationships may be more intuitively expressed diagrammatically, where it can be seen that the amplitude of the Coriolis spiraling induced by gravity varies exponentially with vessel rotation speed in an inverse manner (Fig. 2*B*).

Examples of Coriolis forces come in diverse sizes. One dramatic example of Coriolis forces in action is the spiraling of a hurricane. A hurricane is a unidirectional wind traveling across the spinning surface of the earth as it rotates. As the wind crosses the spinning globe, Coriolis forces induce the characteristic hurricane spiraling that is so familiar on weather maps (71).

The second tradeoff limiting the efficacy of the rotating-wall vessel occurs, in contrast to the exponential relationship between rpm and gravitational vectors, as centrifugal force is directly linearly proportional to rotation speed (Fig. 2*B*) (20, 37, 50, 72). Several practical applications of these relationships explain the behavior of particles in the rotating-wall vessel (72). At low rotation speeds, the gravity-induced deviations predominate and the spirals are extremely large, with many wall impacts. At its extension, at extremely low rotation speeds, as the rotations approach zero the spirals are so large that the particles make one nearly continuous wall impact, by sitting on the bottom of the vessel. At high rotation speeds, centrifugal forces dominant distortions, wall impacts again rise, and the particles stick to the outer wall of the vessel. These effects define the practical operating range for rotating-wall vessels. As larger particles, with correspondingly larger sedimentation rates, are cultured, we become less able to choose a rotational rate that minimizes wall impacts or contact.

Even more important than these boundary conditions are the limitations on particle size mediated by these gravity-dependent changes (20, 37, 50, 72). At low rotation speeds, increasing the rotation speed of the vessel (Fig. 2*B*, *left*, where gravitational effects are dominant) can minimize the deviations from an ideally circular course of the particles. As the particles increase in size, the terminal velocity increases, and more shear is required to keep the particles in suspension. Continued improvement due to reduction of gravitational effects as vessel rotation speed increases is offset and outstripped by increasing centrifugal effects (Fig. 2*B*, *left* to *right*, showing increasing rotation speed). As particles such as cell aggregates increase in size, high rpm is needed to keep them in suspension, and centrifugal forces become dominant in deviation from a perfectly circular rotation path. Wall impacts increase due to necessary increases in angular velocity, as well as an exponential increase in shear with increasing particle radius (*Eq. 1*). These limitations defined by the rotation required to suspend increasingly large particles in culture, combined with exponentially increasing shear with particle radius, define a limit to the unit gravity operation of the rotating-wall vessel for suspension culture. We observe significant effects as typical tissue aggregates approach 1 cm in diameter (72, 73).

The second tradeoff in the rotating-wall vessel is that rotation speed needs to be optimized by finding the angular velocity at which the total of Coriolis and centrifugal forces is minimized. Note carefully that both the Coriolis and centrifugal forces acting on particles in the vessel are directly related to the terminal velocity. If terminal velocity is minimized by changes in the mechanical culture conditions, the Coriolis and centrifugal forces are simultaneously minimized. However, only the gravity-induced motions reach steady state. The centrifugation and secondary, very small

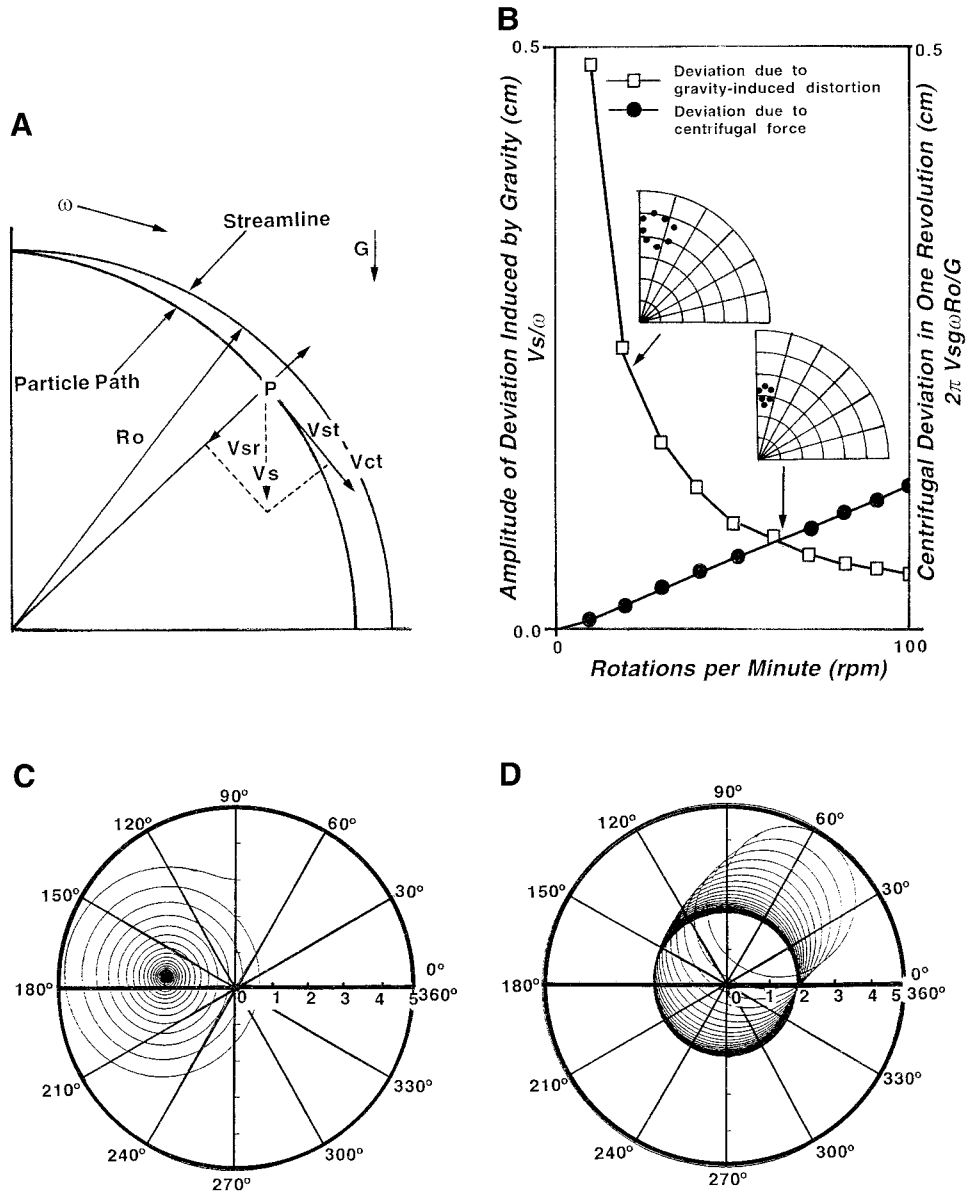

Fig. 2. Operating parameters and limits to rotating-wall vessel function. *A*: vector velocity diagram. The forces acting on a particle under the influence of a rotating viscous fluid are shown. Gravity-induced sedimentation (V_s) can be resolved into inwardly directed radial (V_{sr}) and tangential (V_{st}) components. There is also an outwardly directed particle velocity due to centrifugal force (V_{cr}). Tangential Coriolis-induced motion (V_{ct}) leads to spiraling of particles in the fluid stream. *B*: magnitude of deviation of particles across streamlines in the rotating-wall vessel. Note that the relationship between gravity-induced distortion and rotations per minute is an inverse exponential function, whereas the deviation due to centrifugal force is directly linearly related to rotations per minute. The radius of Coriolis-induced spiraling at low speed (*left inset*) can initially be reduced by increasing rotation speed but rapidly degenerates (*right inset*) until cell aggregates are stuck to the outer wall as the rate of rotation increases (moving from *left* to *right* across the diagram; redrawn from Ref. 72). *C*: calculated trajectory of a particle in the rotating-wall vessel as a function of time in a stationary frame of reference. Note particle advances to asymptotic steady state, as balance point of centrifugal and Coriolis forces is approached. *D*: calculated trajectory of rotating-wall vessel cell aggregate drawn in rotating frame of reference. *C* and *D* are redrawn from Ref. 20 (© Carl Hanser Verlag; reprinted by permission).

Coriolis effects are ever present, causing a slow drift outward and a phase lag with respect to the vessel wall. Within the operation range of the vessel, we usually do not observe the centrifugation outward or Coriolis effects because, in practice, other disturbances and fluid motions, such as motor vibrations, mask them.

To demonstrate the functioning of the vessel, Fig. 2*C* depicts the trajectory of a cell aggregate in the vessel using a stationary frame of reference. The aggregate spirals into a steady-state position in which the tradeoff of Coriolis and centrifugal forces is balanced. The actual particle trajectory during the turning of the rotating-wall vessel is shown in Fig. 2*D*. Hence, within a few rotations, particles come to a steady-state trajectory, in which the majority of the time they are close to the center of the fluid annulus of the vessel.

The interrelationships of several major determinants of terminal velocity and shear in the rotating-wall vessel can be summarized in a diagram (Fig. 3). Both terminal velocity and shear are directly proportional to the difference in density between particles in the vessel and the fluid media (Fig. 3*A*). Neither terminal velocity nor shear is affected at all by rotation speed (Fig. 3*B*) (although rotation speed determines the number of wall impacts). Particle radius has a direct linear effect on terminal velocity and a more complex curvilinear effect on shear (Fig. 3*C*). Media viscosity has a complex curvilinear effect on terminal velocity but no effect on shear (Fig. 3*D*).

Tradeoff III: Mass Transfer

The third tradeoff in the vessel is that changes in mechanical culture conditions also affect nutrient delivery to cells by convective bulk flow and diffusion. The delivery of nutrients to a cell in suspension culture depends on two factors: the "convective" motion of

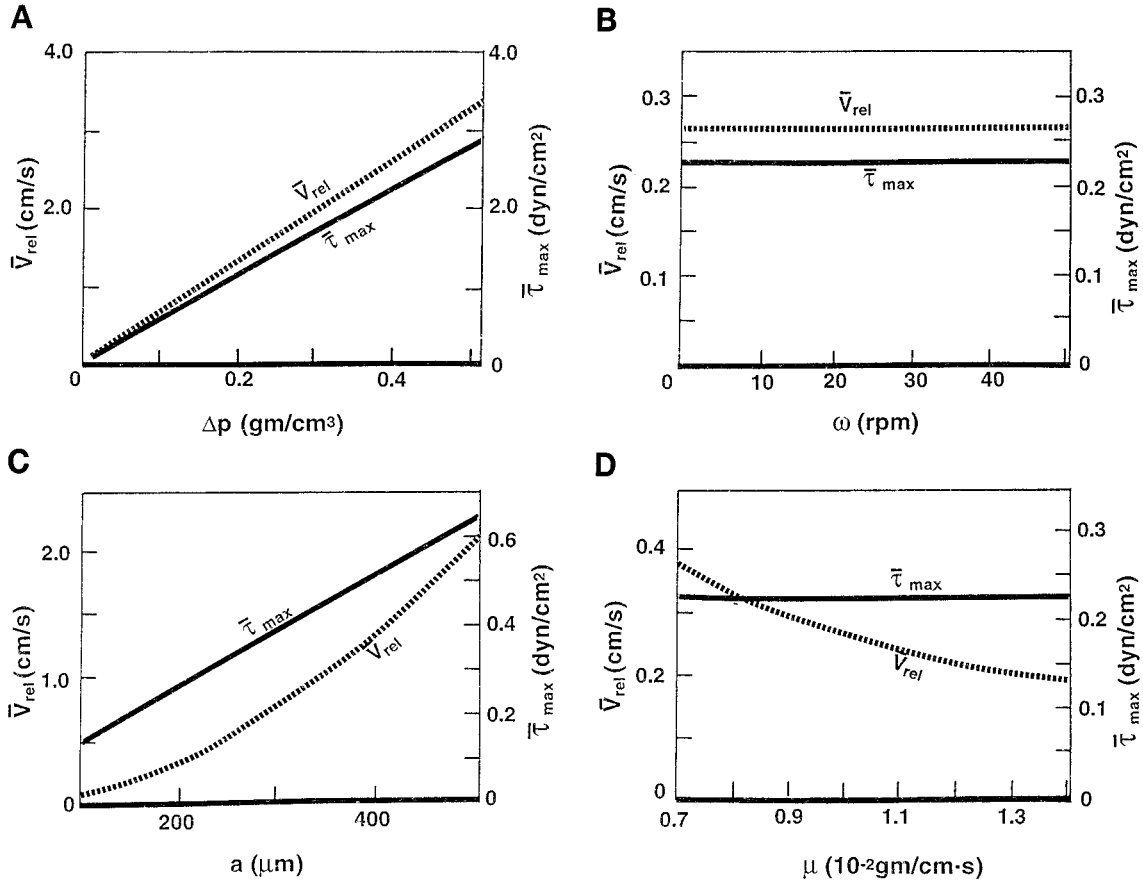

Fig. 3. Terminal velocity ($\bar{V}_{rel}$); and maximum shear stress ($\bar{\tau}_{max}$) for a microcarrier cell aggregate as a function of density ($\Delta\rho$; *A*), rotation speed (ω; *B*), particle radius (*a*; *C*), and viscosity of culture medium (μ; *D*). Terminal velocity and maximum shear are plotted on the same axes to emphasize the relationship, i.e., the differences between the determinants of these 2 mechanical culture conditions. See the text for a full description. The figure is redrawn from Ref. 20 (© Carl Hanser Verlag; reprinted by permission).

nutrients depends on the relative flow of nutrients and movement of the cell and on the diffusion rate of each solute in the particular culture medium (11, 19, 35, 60, 71).

In conditions where there is no relative fluid motion (that is, terminal velocity approaches 0), diffusion becomes the dominant mechanism for mass transport. The diffusional flux of a component in the *z*-direction ($J_{A,z}$) is given as the product of the diffusion coefficient of that species (D_{AB}) and the concentration gradient (11, 19, 71)

$$J_{A,z} = -D_{AB}\frac{dC_A}{dz}$$

The diffusion coefficient, D_{AB}, is generally defined for a particular solute (A) in a particular solvent (B). Liquid diffusivities are typically on the order of 10^{-5} cm^2/s and are inversely proportional to both the molecular size of the solute (r_A) and the viscosity of the solvent (μ_B) (11, 19, 71)

$$D_{AB} = \frac{\kappa T}{6\pi r_A \mu_B}$$

The equation predicts, for example, that in an aqueous solution of 70-kDa dextran, with larger size and viscosity compared with 10-kDa dextran, there would be reduced diffusion of dissolved species such as glucose. In the case of limited mass transport, there is the possibility of limited nutrient availability and toxic waste accumulation in the microenvironment around a cell. Thus in the extreme, where terminal velocity approaches zero for a cell in suspension culture such as in the rotating-wall vessel, there may be limited growth or even cell death (11, 19, 71). The equations immediately above also predict that reducing the viscosity of the culture medium and/or providing a chemical gradient of the solute can relieve mass transport limitations.

The ratio of the convective motion to the diffusion rate is a dimensionless constant known as the Peclet number, which is highly predictive of mass transport limitations. For small particles, around the size of bacteria, with a Peclet number <10, diffusion can be limiting in suspension culture. For mammalian cells on beads with a Peclet number >10, mass transport tends to be convection, not diffusion, limited (11, 19, 35, 64, 71). In other words, a cell aggregate falling through the

culture media in the rotating-wall vessel is fed as it effectively sieves through the nutrient media. It is only when the motion of cell aggregates and nutrients becomes equal, as terminal velocity approaches zero, that there is any role for diffusion in nutrition.

The practical application of the mass-transfer tradeoff is twofold. First, cell viability must be monitored in suspension culture to ensure that nutrient delivery has not become limiting. Second, this tradeoff suggests that it is better, when terminal velocity is reduced by driving particle and culture medium densities together, to utilize lighter microaggregate beads whenever possible rather then to increase the density of the culture media. Density increases also increase viscosity at the price of less diffusion, a metabolic cost avoided when the problem is solved by the use of lighter beads.

VALIDATING THE MATHEMATICAL MODELS: OBSERVED VS. PREDICTED EFFECTS OF MECHANICAL CULTURE CONDITIONS ON PARTICLE MOTION

Importantly, the predicted particle pathways in rotating-wall vessel culture suggested by these relationships have been tested and validated experimentally (50, 73). NASA optimally engineered the low-shear, laminar flow conditions in the rotating-wall vessel in an attempt to develop a robust model system with many features of the low-shear, low-turbulence conditions predicted for cell culture in space. Hence many of the experimental validations of these principles have been based on changing gravitational conditions. Practical confirmation of the effects of gravity were obtained by video recording and quantitating the path of particles in a rotating-wall vessel during variations induced by the parabolic flight path of a NASA KC-135 aircraft. Popularly referred to as the "vomit comet," this aircraft has been utilized to provide repeated ~25-s periods of <0.1 gravity by following a looping flight path. The predicted and observed particle motion of beads at different forces in the rotating-wall vessel agreed to within <1%, and sources of error such as irregularities in the rotator drive motor speed or secondary fluid motions caused by particle motion were identified (72).

Robeck (50) wrote a computer simulation of this model and tested it against video data of cells growing in the rotating-wall vessel in microgravity on the space shuttle on space transportation system flight STS-70. There was excellent agreement between the predicted and observed particle paths in this experiment.

OTHER WAYS OF DISRUPTING OPTIMAL CULTURE FLUID DYNAMICS

The optimized mechanical culture conditions can be disrupted in many other ways, such as differential rotation of inner and outer walls of the vessel or bubble formation. These disruptions are remarkable in that an apparently tiny change in fluid flow can have dramatically disproportionate effects on shear and turbulence (22).

Differential Rotation

To study the effects of reintroduction of shear in a structured quantifiable manner, it is desirable to be able to reinduce shear in the rotating-wall vessel. One simple modality to reinduce shear is to rotate the inner coaxial oxygenator (inner wall) and the outer wall at different speeds. This differential rotation has been used to reintroduce quantifiable levels of shear (22, 57). Counterrotation of inner and outer walls, with or without a mixing vane, can also be used to mix the contents of the vessel, but this incurs high amounts of shear. Differential rotation is potent in its ability to induce shear. This is where the real laminar flow gradient comes in and thus the specifically induced shear forces. In the case where the inner and outer angular velocities are equal, the fluid particles in the medium, once in equilibrium, should be rotating at essentially the same rotations per minute as the rotating-wall vessel itself. The major determinant of the amount of shear ($\sigma_{r\theta}$) induced is the difference in rotation speed of the inner (Ω_i) and outer walls (Ω_o) [22], the ratio of the inner (k_R) and outer (R) wall radii, and constants characteristic of

$$\sigma_{r\theta} = -m\left(\frac{R}{r}\right)^2\left[\frac{2(\Omega_i - \Omega_o)}{n\left[\left(\frac{1}{k}\right)^{2/n} - 1\right]}\right]^n$$

a particular fluid (m and n).

Both theoretical and experimental approaches have validated the dynamic ranges of inner and outer wall rotation, together with associated shear stresses, which can be achieved before laminar flow degrades (1). Diverse forms of turbulence occur as laminar flow degrades, although the range of relative wall rotations at which laminar flow is preserved is predictable and has been simply summarized in both mathematical and diagrammatic formats (1).

Bubbles

The optimized engineering of the rotating-wall vessel in which shear and turbulence are minimized is entirely dependent on maintaining laminar flow in the culture vessel. These are several examples of simple perturbations, which induce turbulence and potently disrupt the advantages and biological efficacy of the vessel. The simplest and best modeled example is the effect of a single small bubble placed on the baseplate of the vessel (6, 36, 46). Such an apparently humble change induces turbulent eddies in the distal third of the vessel, with the exact pattern of turbulence heavily dependent on the size of the bubble (46). However, there are simple ways to prevent bubble formation by using suitably humidified dissolved gas mixtures.

BEADS AND OTHER SCAFFOLDS

Many cells such as lymphocytes or bacteria can be grown in the rotating-wall vessel in suspension with no other support (10, 41, 57). Adhesion-dependent cells

such as renal epithelial cells or prostate epithelial cells, which require an adherent base to proliferate, are commonly grown on beads (24, 25, 76). The most popular bead is a 175-μm-diameter collagen bead known as Cytodex-3 with specific gravity of 1.04. Beads manufactured of collagen and glass are commercially available in a variety of diameters and densities, allowing for simple modulation of the initial mechanical conditions. As all of these beads are difficult to impossible to section, structural studies other than scanning electron microscopy are often performed on Cultisphere gelatin beads, which are a porous honeycomb that is simple to section (24, 68). Similarly, some cells, such as cartilage, grow well on dissolvable polyglycolic acid matrices (17), which degrade completely over a matter of days in solution.

CELLULAR AND MOLECULAR MECHANISMS OF ROTATING-WALL VESSEL ACTION

An understanding of the engineering principles of suspension culture is helpful, but for a cell biologist the molecular mechanisms by which the cellular changes are mediated are more interesting. There are precedents in the cell culture literature by which physical forces can induce changes in cell metabolism and function. Heat shock proteins, in which tiny changes in temperature induce a cascade of genetic and protein changes, are a well-documented example (5, 17, 74). More recently, there is abundant evidence than shear stress due to flow across the vascular endothelium is critical to maintaining many attributes of normal cell structure and function (21, 47, 48, 66).

The mechanisms by which mechanical culture conditions affect gene expression, protein content, and the structure and function of diverse cell lines is only beginning to be examined systematically. The simplest explanation of the transduction of gravity-dependent effects occurs in cells with sedimenting organelles such as otic hair cells or statoliths in plants. The altered mechanical culture conditions of spaceflight have dramatic effects on cell growth and metabolism, as well as the production of bioproducts from growth hormone to hybridomas (reviewed in Refs. 33, 60 and 61). Postulated mechanisms include cytoskeletal elasticity changes, electrical and chemical signaling, and channel activation (60–65), although there has been no systematic experimental dissection of these mechanisms to date. There are, however, a few experimental clues as to mechanism, including a role for shear stress (32), gene array investigation of patterns of genetic response (24, 25, 32), definition of some signal transduction changes (9, 37), and modeling of the effects of the vessel on both the cell cycle (28), and pathways of cell death (11).

Shear Stress

Fluid shear stresses generated by blood flow in the vasculature can profoundly influence the phenotype of the endothelium by regulating the activity of certain flow-sensitive proteins (enzymes, for example) as well as by modulating gene expression (21, 47, 48, 66). The finding that specific fluid mechanical forces can alter endothelial structure and function has provided a framework for a mechanistic understanding of flow-dependent processes, ranging from vascular remodeling in response to hemodynamic changes to the initiation and localization of chronic vascular diseases such as atherosclerosis (21, 66).

The effects of shear stress on vascular endothelial cells raised obvious questions about similar responses in other cell types. Our laboratory provided evidence that shear stress response elements, which modulate gene expression in endothelial cells, are also active in epithelial cells (32). Rotating-wall culture of renal cells changes expression of select gene products, including the giant glycoprotein scavenger receptors cubulin and megalin, the structural microvillar protein villin, and classic shear stress response genes ICAM, VCAM, and MnSOD. With the use of a putative endothelial cell shear stress response element binding site as a decoy, the role of this sequence in the regulation of selected genes in epithelial cells was demonstrated (32).

Gene Expression

Several lines of evidence suggest that physical factors can change cellular gene expression, but no one had addressed the extent or nature of these changes quantitatively (24, 25). To elucidate the specific mechanical parameters responsible for the genetic and morphological changes, we grew primary human renal cell cultures under conditions providing differences in three-dimensionality, cospatial relationship of cells, and shear (24, 25) (Table 3). Each culture derived from the same stock of human renal primary cells was grown for 6 days. The rotating-wall vessel was the best ground-based optimization of the mechanical parameters. To fully optimize these mechanical parameters, aliquots of the cells were grown in space on the space shuttle, with fixation on *day 6*. Culture of the cells during exposure to a force of three times the earth's gravity, achieved by placing culture bags in a centri-

Table 3. *Cell culture modalities and mechanical culture conditions*

Cell Culture Type/ Mechanical Condition	3-Dimensionality	Shear and Turbulence	Cospatial Relationship of Dissimilar Cell Types
Rotating-wall vessel	Best current ground-based	Best current ground-based	Best current ground-based
Microgravity	Optimal	Optimal	Optimal
3-g Centrifuge	Unchanged	Unchanged	Unchanged

fuge, was used as a negative control as none of the mechanical conditions hypothesized to be relevant were modulated. Gene expression analysis using microarray technology allows detailed observations of genetic expression under various culture conditions, with quantitative comparison of the nature, grouping, and extent of changes.

More than 1,632 genes changed more than twofold at 6 days during microgravity culture on space transportation system flight STS-90 "Neurolab." These changes were not known shear stress response elements and heat shock proteins (shown in green in Fig. 4). Specific transcription factors (shown in red), including the Wilms tumor zinc finger protein and the vitamin D receptor, underwent large changes in microgravity (full data set at web site http://ww.tmc.tulane.edu/astrobiology/microarrays). In the rotating-wall vessel, 914 genes changed expression relative to the bag control. Those genes that changed most on the microgravity array are randomly spread throughout the rotating-wall vessel array, demonstrating that the changes in gene expression observed in microgravity are unique and not just an extension of the rotating-wall vessel. Only five genes changed more than threefold during 3-*g* centrifugation (24, 25).

An escape from the tradeoff of balancing forces by examining the gravity-limited phenomenon of suspension culture in true microgravity demonstrated a dramatic degree of change in gene expression. Microgravity studies suggest unique mechanisms of tissue differentiation, which are distinct from the present best ground-based simulations (24, 25).

Signal Transduction

There are select phenomena in which specific signal transduction elements active during rotating-wall vessel culture have been defined. Activation of purified T cells with cross-linked CD2/CD28 and CD3/CD28 antibody pairs were completely suppressed in the rotating-wall vessel, suggesting a defect in signal transduction (10). Activation of purified T cell protein kinase C (PKC) with phorbol 12-myristate 13-acetate or calcium-dependent ionomycin activation were unaffected by rotating-wall vessel culture. Furthermore, submitogenic PKC-dependent doses of phorbol 12-myristate 13-acetate alone but not ionomycin alone restored phytohemoagglutinin responsiveness of peripheral blood mononuclear cells in rotating-wall vessel culture. These data indicate that during polyclonal activation the signaling pathways upstream of PKC activation are sensitive to mechanical culture conditions.

PC12 cells cultured under "simulated microgravity" conditions showed altered patterns of protein tyrosine phosphorylation and prolonged activation of c-fos, a member of the AP-1 nuclear transcription factor complex (39).

Apoptotic/Necrotic Pathway Monitoring

To more fully understand the effects that rotating-wall vessel cultivation has on cellular longevity, the mode and kinetics of *Spodoptera frugiperda* cell death was studied and modeled in the quiescent environment of the rotating-wall vessel and compared with a shaker-flask control (11). Data from flow cytometry and fluorescence microscopy show a twofold greater accumula-

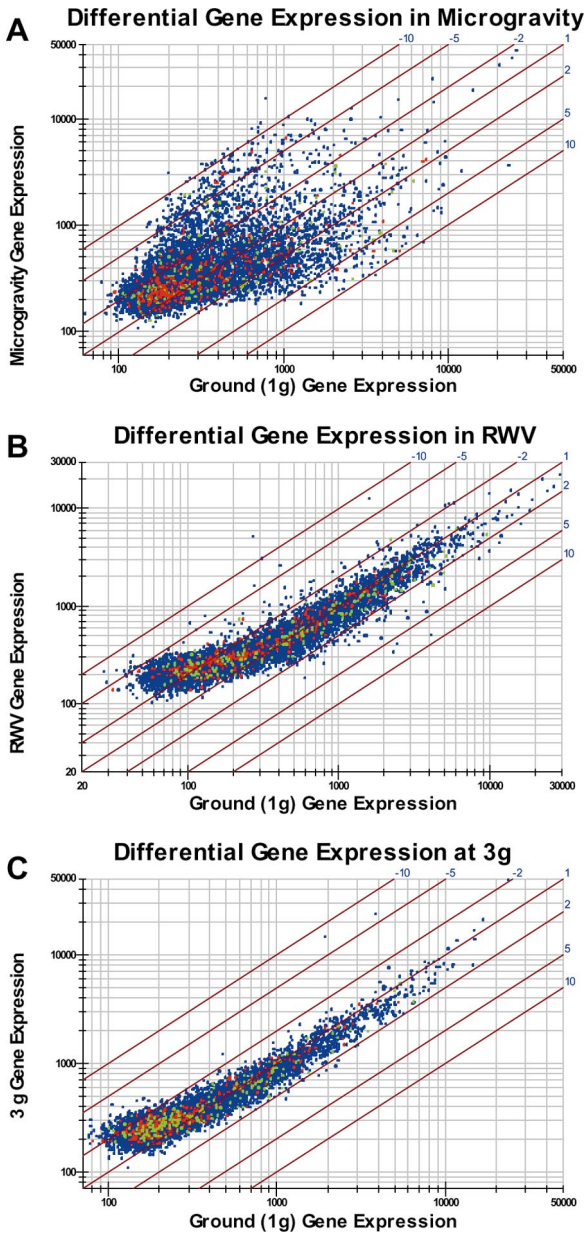

Fig. 4. Gene expression and mechanical culture conditions. Human renal cortical cells were cultured in DMEM/F-12, 10% fetal calf serum, on Cytodex-3 beads. After 6-day cultures, samples were fixed with Omnifix and stored at 7°C. RNA was selected with biotinylated oligo (dT), separated with streptavidin paramagnetic particles, and reverse transcribed with fluorescent bases, and a competition binding analysis was performed on microarrays (Incyte, Palo Alto, CA). Microgravity, 3-*g* centrifuge, and rotating-wall vessel are each compared with a static nonadherent bag culture grown in parallel. In *A-C*, 10,000 genes are represented by individual dots. Shear stress proteins and heat shock proteins are shown in green, and transcription factors in red. Genes with similar expression lie on a line from the origin to the *top right-hand* corner (labeled 1) with expression level reported by distance from the origin. The scale on each axis is log to the base 2. The figure is reproduced from Ref. 24.

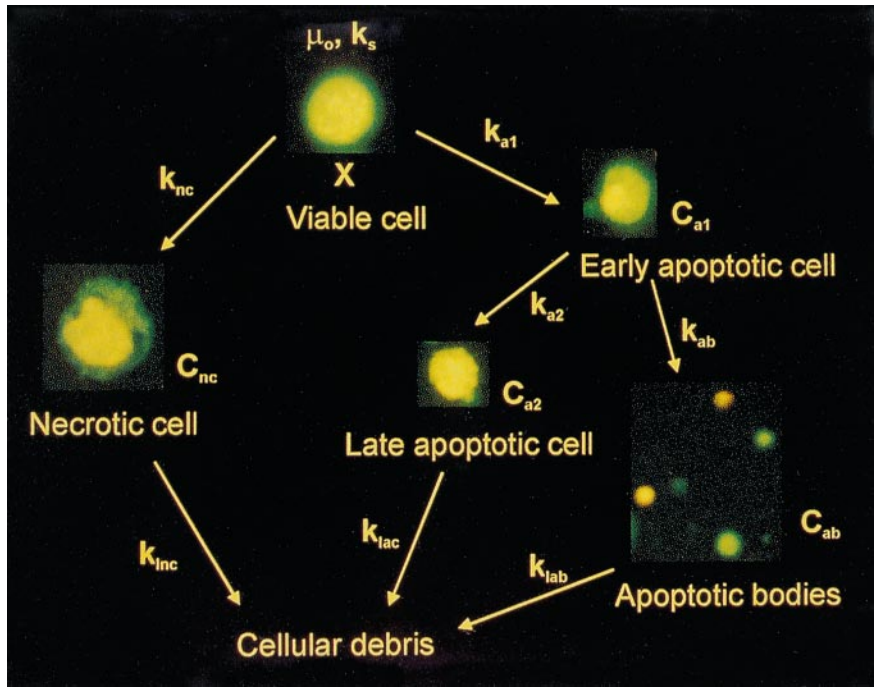

Fig. 5. Modeling of apoptosis/necrosis. Depicted are stages of bimodal cell death showing nomenclature for cell concentration (C) and rate constants (k) used in a kinetic model. Cell species are distinguished by their appearance under the fluorescent microscope. Primary and secondary necrotic nuclei appear bright orange from ethidium bromide staining. They can be distinguished on the basis of the diffuse staining pattern of primary necrotic DNA vs. the highly condensed or fragmented DNA of secondary necrotic (late apoptotic; lac) cells (nc). Viable and early apoptotic nuclei both appear bright green due to acridine orange staining, but the former has an organized structure whereas the latter appears highly condensed or fragmented. Apoptotic bodies (ab), as distinguished from general debris, appear as tiny spherical objects, either bright green or orange. These data mark the beginning of quantitative modeling of the cell death pathways in the rotating-wall vessel. The figure is reproduced from Ref. 11 (© Wiley-Liss, Inc., reprinted by permission).

tion of apoptotic cells in the rotating-wall vessel culture (Fig. 5). Kinetic constants reveal that total cell death is reduced in the rotating-wall vessel, and the accumulation of apoptotic cells in this vessel results from reduced depletion by lysis and secondary necrosis. The ratio of early apoptotic to necrotic cell formation is found to be independent of culture conditions. In the model, apoptosis is only well represented by an integral term, which may indicate its dependence on accumulation of some factor over time; in contrast, necrosis is adequately represented with a first-order term. Cell-cycle analysis shows that the percentage of tetraploid cells gradually decreases during cultivation in both vessels. This suggests the tetraploid phase as the likely origin for apoptosis in these cultures. These studies provide a means for further enhancing culture longevity.

These findings in the rotating-wall vessel are consistent with other model systems (75). When six hybridoma cell lines grown under diverse experimental conditions in both normal continuous and perfusion cultures are analyzed with respect to the significance of nutrients and products in determining the growth and death rates, the specific growth rate (μ) almost linearly correlates with the ratio of the viable cell concentration (N_V) to the dilution (perfusion) rate (D). Similarly, the specific death rate (k_d) is a function of the ratio of the total cell concentration (N_t) to the dilution (perfusion) rate, which strongly suggests the formation of not yet identified critical factors or autoinhibitors that determine both the growth and death rates of hybridoma cells. That is, the cells produce a bioproduct that accumulates in the culture medium and effects changes in cell cycle and cell death in a concentration-dependent manner.

Role of the Cytoskeleton

The central role of the cytoskeleton in cell scaffolding, load bearing, and transportation of vesicles, continues to make it a popular candidate for transducing physical stimuli into other cellular processes (40, 41, 64). There is some molecular evidence for this process, including concomitant cytoskeletal alterations characterized by diffuse, shortened microtubules, increased apoptosis, and time-dependent elevation in Fas/APO-1 protein in space-flown human lymphocytes (40, 41).

OTHER BENEFITS OF OPTIMIZED ROTATING-WALL VESSEL CULTURE: COCULTURE, NUTRIENT DELIVERY, AND EASE OF REAGENT EXPOSURE

The rotating-wall vessel provides some other advantages relevant to many forms of tissue culture. The vessel supports coculture efficiently by bringing different cell types of different size and density together simply and efficiently. It is very simple to expose the suspension cultures to reagents in the vessel. These properties of the vessel are especially important for tissue engineering studies, one of the areas in which utilization of the rotating-wall vessel has been most active. The rotating-wall vessel has been successful in engineering prostate organoids (49, 76), colon carcinoma (28), and cartilage (18, 69), among other tissues (68).

NOMENCLATURE: SIMULATED MICROGRAVITY VS. OPTIMIZED SUSPENSION CULTURE

As the rotating-wall vessel was developed by NASA to simulate, as far as possible, culture conditions predicted to occur during experiments in space, these

conditions were given the moniker simulated microgravity (22, 72). Many articles have been published as "the effects on simulated microgravity on. . . ." Without one's knowledge of the purpose and history of this term, it tends to be confusing, as it suggests the physical impossibility that there is no gravity inside the vessel. These reviewers strongly advocate a return to descriptive, intuitively informative nomenclature: the rotating-wall vessel changes mechanical culture conditions to provide a form of suspension culture optimized for low shear and turbulence, not simulated microgravity.

CONCLUSIONS

To minimize mechanical damage to cultured cells, suspension cultured should be performed under conditions of near-solid body or laminar flow. This is most simply achieved in a solid-body rotation Couette-flow, zero-head-space culture vessel such as the rotating-wall vessel. The rotating-wall vessel provides fluid dynamic operating principles characterized by *1*) solid body rotation about a horizontal axis that is characterized by colocalization of cells and aggregates of different sedimentation rates, optimally reduced fluid shear and turbulence, and three-dimensional spatial freedom; and *2*) oxygenation by diffusion. The utility of turbulent flow culture devices such as roller bottles or stirred fermentors is limited by the severe mechanical stresses they induce.

Optimization of suspension culture is achieved by application of three tradeoffs. First, terminal velocity should be minimized by choosing microcarrier beads and culture media as close in density as possible. Reducing or removing gravity, which likely underlies many of the effects of spaceflight on cell culture, can also minimize terminal velocity.

Second, rotation in the rotating-wall vessel induces Coriolis and centrifugal forces. Both of these forces are directly dependent on terminal velocity and are minimized as terminal velocity is minimized.

Third, mass transport of nutrients to a cell in suspension culture depends on both terminal velocity and diffusion of nutrients. As terminal velocity is minimized, nutrient delivery by mass flow is also reduced. Diffusion depends on the properties of both the individual nutrient and the culture medium. For small particles such as bacteria, diffusion can limit nutrient supply in suspension culture, but for large particles such as mammalian cells on microcarrier beads mass transit is likely to be terminal velocity limited. Both these limitations to nutrient supply can be overcome by lowering the viscosity of the culture medium and/or providing a nutrient supply to form a concentration gradient. Introduction of differential vessel component rotation rates or incorporation of devices such as the viscous pump (36) allows induction of arbitrary levels of active mixing with highly controlled and homogenous fluid mechanical conditions. This is particularly important in spaceflight, where particle sedimentation is absent.

Several lines of evidence support a role for multiple molecular mechanisms in the transduction of mechanical culture conditions into cellular effects. These mechanisms include effects of shear stress, changes in cell cycle and cell death pathways, and upstream regulation of secondary messengers such as PKC. New methods of gene array analysis are beginning to dissect these mechanistic possibilities.

Finally, the discipline of suspension culture needs a systematic analysis of the relationship between mechanical culture conditions and biological effects, with an emphasis on cellular processes important for the industrial production of biological pharmaceuticals and devices.

The application of suspension culture has been limited by the failure to develop a simple optimization strategy based on mechanical culture conditions. This review may provide a roadmap to facilitate both industrial and academic applications to help fulfill the massive promise of suspension culture.

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