Expression of renal cell protein markers is dependent on initial mechanical culture conditions

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Cowger, Nancy L., Edmund Benes, Patricia L. Allen, and Timothy G. Hammond. Expression of renal cell protein markers is dependent on initial mechanical culture conditions. J Appl Physiol 92: 691–700, 2002.—The rotating wall vessel is optimized for suspension culture, with laminar flow and adequate nutrient delivery, but minimal shear. However, higher shears may occur in vivo. During rotating wall vessel cultivation of human renal cells, size and density of glass-coated microcarrier beads were changed to modulate initial shear. Renal-specific proteins were assayed after 2 days. Flow cytometry antibody binding analysis of vitamin D receptor demonstrated peak expression at intermediate shears, with 30% reduction outside this range. Activity of cathepsin C showed the inverse pattern, lowest at midshear, with twofold increases at either extreme. Dipeptidyl-peptidase IV had no shear dependence, suggesting that the other results are specific, not universal, changes in membrane trafficking or protein synthesis. On addition of dextran, which changes medium density and viscosity but not shear, vitamin D receptor assay showed no differences from controls. Neither cell cycle, apoptosis/necrosis indexes, nor lactate dehydrogenase release varied between experiments, confirming that the changes are primary, not secondary to cell cycling or membrane damage. This study provides direct evidence that mechanical culture conditions modulate protein expression in suspension culture.

suspension culture; vitamin D receptor; tissue engineering; cell culture

To optimize mechanical culture conditions in suspension culture vessels, the challenge is to minimize shear and turbulence, while keeping the cell aggregates in suspension and preserving mass transport of both nutrients and gases (10, 11, 13, 22, 27, 37). These requirements are embodied in the rotating wall vessel (RWV): a horizontally rotating cylindrical culture vessel with a coaxial tubular oxygenator (13, 37).

The RWV has characteristic features that determine its utility. First, the culture medium is gently mixed by rotation, avoiding the necessity for stirring vanes that can damage cells with local turbulence. Second, fluid flow is laminar. The outer vessel wall, the coaxial oxygenator, and the fluid inside all rotate at the same slow (10–60 rpm) rate, avoiding shear from differential rotation. Third, there are no bubbles in the vessel because oxygenation is by diffusion across a silicone membrane (13, 22, 37). Interaction with bubbles is typically a potent source of cell damage (3, 37). In the RWV, three-dimensional assembly and colocation of dissimilar cell types are accommodated. The result is spheroid formation with increased cell-cell and cell-matrix interactions and tissue differentiation (4, 5, 13, 35). Aggregates of human prostate tumor cells, for example, had stronger staining for select cytoskeletal proteins, suggesting a more differentiated population than control cells grown in spinner flask or as a monolayer (4).

The question now becomes whether the engineering optimization of shear to extremely low levels reflects biological optimization as well. Several lines of evidence suggest not only that many mammalian cells live in a milieu of shear but that shear stress is necessary for normal structure and function of these cells (12, 20, 25, 28, 29). Perhaps the best studied example of shear dependence of gene and protein expression is the serial clusters of genes expressed during exposure of vascular endothelial cells to shear stresses that mimic flow in blood vessels (20, 25, 28, 29).

Kidney cells in vivo, especially renal proximal tubular cells, also experience a shear stress environment.

Most differentiated cells from diverse tissue sources lose their specialized features and “dedifferentiate” when grown as a two-dimensional monolayer culture (1, 13, 35). This greatly limits the utility of these cultures for the study of tissue-specific receptors, metabolic pathways, signal transduction mechanisms, and/or nuclear transcriptional events. The classic cell biology approach to overcome this problem and maintain many differentiated features of cells in culture is to grow the cells in suspension culture (1). Suspension culture can be performed in a diverse array of culture vessels, often including beads or other matrixes to provide support for adherent cells (1, 13, 35, 37).

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far larger than the levels present during optimized RWV culture (14). Shear forces vary from ~1 to 5 dyn/cm² over the normal range of flow rates in the proximal tubule (14), whereas shear is estimated at 0.5 dyn/cm² or less for the RWV (13). Three recent studies from our laboratory have demonstrated the importance of mechanical culture conditions on gene and protein expression for human renal cells (16, 17, 20). Kayser et al. (20) compared RWV cultures to stirred and static controls. The findings include the following: 1) tissue-specific markers such as megalin protein and mRNA and also cubilin protein expression increased dramatically in the RWV; 2) villin, the structural protein of microvilli, showed an early mRNA increase in association with microvillar reformation; and 3) MnSOD, a shear-stress response gene, showed an early mRNA decrease. Hammond et al. (16, 17) documented large populations of genes influenced by mechanical culture conditions by gene array analysis of 6-day steady-state human renal cell expression of 10,000 known genes. More than 800 genes changed at least twofold during RWV culture, and more than 1,600 genes changed as shear approached zero in the microgravity of space (16, 17).

Although a relationship between mechanical culture conditions and renal cell gene and protein expression is established, no strict correlation has been made in a controlled culturing environment. In the current study, individual properties of suspension culture are varied, specifically medium density, and size or density of microcarrier beads, within the single environment of a RWV. As a result, the shear stress for cell aggregates is varied within a small dynamic range. Our study assists in answering the following questions: Which common renal cell proteins respond to dynamic culture conditions? Are the cellular responses highly specific? Is there an in vivo correlation for this response?

MATERIALS AND METHODS

General cultivation. Clonetics (San Diego, CA) isolated human renal cortical cells from kidneys unsuitable for transplantation (15, 20), and the cell fractions consist of a natural mixture of cells from the renal cortex. Flow cytometry analysis of the proximal tubular marker leucine aminopeptidase demonstrated that the cells are >98% proximal tubular cells after culture in selective media (data not shown). These cells were stored frozen at passage 2 and, a few weeks before an experiment, were thawed and brought up to passage 4 as monolayer cultures. Experiments were conducted in RWVs, specifically the 55-ml slow-turning lateral vessel (STLV) model, manufactured by Synthecon (Houston, TX). The STLV has a cylindrical chamber (~6-cm diameter by 2-cm length) with gases exchanged across a silicone rubber membrane mounted on a smaller cylindrical core. Stationary control cultures, included in some of the experiments, were grown in 100-ml silicone SiCulture bags (TC Tech, Minneapolis, MN). Cultivation was in a 37°C incubator with a 5% CO₂ humidified atmosphere.

Culture conditions. The culture medium was DMEM/F12, pH 7.4, supplemented with 30 mM sodium bicarbonate and 30 mM HEPES (total), 10% fetal calf serum, and an antibiotic cocktail (Ciprofloxacin and Fungizone) (15, 20). For the majority of experiments presented here, renal cells were inoculated at ~3.5 × 10⁶ cells/ml attached to glass-coated microcarrier beads (SoloHill, Ann Arbor, MI) at 9–18 mg/ml but at the same cell coverage of 8 × 10⁵ cells/cm² bead surface area (see Discussion). Experiments were conducted for 2 days in the STLV, rotating at 17 rpm, or in the stationary bag. This time period was considered long enough to observe differences in aggregation and gene and protein expression while not requiring feeding (confirmed by satisfactory pH and glucose values at harvest). Bead properties were varied between experiments, using the following average diameters (d_avg) and densities (ρ): d_avg = 120 μm (ρ = 1.02, 1.03, and 1.04 g/ml); d_avg = 180 μm (ρ = 1.04 g/ml); d_avg = 275 μm (ρ = 1.04 g/ml). When the larger beads are used for cell culture experiments, the same bead concentration cannot be used throughout because the cell-to-bead ratio will increase. Instead, we chose the constant parameter as bead coverage. The same value of cells per square centimeter should result in the same potential for interactions between cells over all experimental conditions.

For an additional set of experiments, 2% (wt/vol) dextran (70 kDa) was added to the medium. Renal cells were inoculated at 7 ± 1 × 10⁵ cells/ml attached to CytoDex-3 (Pharmacia, Uppsala, Sweden) microcarrier beads at 2.5 mg/ml. The CytoDex-3 beads, made of collagen-coated dextran, have a density of 1.04 g/ml and an average diameter of 175 μm. These experiments were conducted for 5 days in the STLV, rotating at 17 rpm. Conditioned medium was replaced with fresh at 25% of the vessel volume on day 2 and 35% of the volume on days 3 and 4. After 5 days, cells were harvested and processed for membrane protein studies.

Calculation of mechanical properties. In mathematically defining the mechanical culture conditions, an initial condition was assumed of cells coating single beads. The terminal velocity (V_t) of a bead inside the RWV was determined by Eq. 1

\[ V_t = \frac{2gR^2(p_b - p)}{9\mu} \]  

(1)

where \( g \) is gravitational acceleration, \( R \) is bead radius, \( p_b \) is bead density, \( p_f \) is fluid density, and \( \mu \) is fluid viscosity.

The density difference between bead and the surrounding fluid (Δp) was varied in our experiments by changing either the density of the bead or the density of the medium. In addition, the bead radius was varied as a separate parameter affecting bead velocity.

The maximum shear stress (\( \tau_{\text{max}} \)) at the surface of a bead is a function of its \( V_t \) as shown by

\[ \tau_{\text{max}} = \frac{3\mu V_t}{2R} \]  

(2)

These two equations allowed us to estimate relative values of shear stress for all of our experimental conditions. These values are shown in Table 1. The equations allowed us to predict a concentration of dextran in the medium that would reduce \( V_t \) with only a minor change in shear.

Statistical analysis. Kolmogorov-Smirnov (KS) summation statistics are often selected as the statistical method of choice for large data sets of independent measurements such as flow cytometry files (38). When KS statistics are applied, the 2,000 independent flow cytometry observations on individual cells or membrane vesicles are treated as separate observations. This greatly increases the statistical power of cell biology data in which experimental replicates have a practical limitation. In addition to KS statistics, single-factor ANOVA was used, as applicable, to compare means between experimental conditions.
ARMS INTERVENTIONS AND SHEAR

Flow cytometry analysis of vitamin D receptor. To quantify the protein content of vitamin D receptor (VDR) on cells grown in the STLV and bag, indirect fluorescent antibody binding was assayed by flow cytometry. Cell aggregates were harvested, washed in phosphate-buffered saline (PBS), and lysed with a PowerGen 125 homogenizer (Fisher Scientific, Pittsburgh, PA). A postnuclear supernatant was prepared by spinning out bead debris and nuclei at 200 g for 10 min in a Beckman ultracentrifuge. Membranes were resuspended in mannitol buffer (300 mM mannitol + 10 mM HEPES, pH 7.4), and nonspecific binding was blocked after treatment with 50% clarified goat serum. Dilutions of rat fluorescent antibody were applied. With samples in fresh mannitol buffer, the membranes were washed, and a goat anti-rat FITC-conjugated antibody was then assayed by flow cytometry. FL2 channel (575 ± 26 nm) was measured with a spectrophotometric end-point kit (Sigma Chemical).

RESULTS

Morphology. The appearance of renal cell aggregates after 2 days culture in the STLV or bag is documented in Table 2. There were obvious differences between the vessel with the largest beads [specific gravity (sg) 1.04] and the other vessels. Aggregates had fewer beads, and there were also cell aggregates not attached to beads. The stationary bag cultures generally averaged larger aggregates, and these were often asymmetrical, even chainlike, compared with more spherical ones in the STLV cultures. Note that small, medium, and large designation for beads refers to those of average diameter 120, 180, and 275 μm, respectively. Any morpho-
logical differences between vessels with 1.03-sg small beads and the 1.04-sg medium beads were subtle.

Transmission electron microscopy demonstrated the presence of microvilli and intracellular desmosomes in each of the RWV cultures examined (Fig. 1). With the exception of dextran in lysosomes in dextran-treated cultures, examination of the images demonstrates no demonstrable differences. In scanning electron micrographs (not shown), cells are flattened as they spread over the bead surface. This likely does not mimic the structural organization of these proximal tubule cells in vivo.

**Cell cycle and apoptosis/necrosis.** Typical results of cell cycle and apoptosis measurements are shown in Table 3. The cell cycle distribution varies little with the experimental condition, with means of $48 \pm 2\% \ G_1$, $48 \pm 3\% \ S$, and $5 \pm 1\% \ G_2 + M$ phase. The percentages of apoptotic and necrotic cells are small and also have limited variation, at $1.7 \pm 0.7\%$ apoptotic and $5.0 \pm 1.3\%$ necrotic.

Table 2. General morphology of bead aggregates

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Condition</th>
<th>Aggregate Size</th>
<th>Aggregate Shape</th>
<th>Unattached Cell Aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>STLV</td>
<td>1.03-sg small glass bead</td>
<td>4–50 beads, most are 10–20 beads</td>
<td>Spherical</td>
<td>Few to none</td>
</tr>
<tr>
<td>STLV</td>
<td>1.04-sg medium glass bead</td>
<td>4–50 beads, most are 10–20 beads</td>
<td>Spherical</td>
<td>Few to none</td>
</tr>
<tr>
<td>STLV</td>
<td>1.04-sg large glass bead</td>
<td>1–4 beads</td>
<td>N/A</td>
<td>Present</td>
</tr>
<tr>
<td>Bag</td>
<td>Combination of the 3 types of glass beads</td>
<td>4–50 beads, some &gt;50 beads</td>
<td>Asymmetrical</td>
<td>Present</td>
</tr>
</tbody>
</table>

Fig. 1. Transmission electron micrographs of renal cortical epithelial cells. A and B: slow-turning lateral vessel (STLV) cultures with 2% dextran added to the medium. Arrows indicate an intracellular desmosome (A) and lysosomes with dextran (B). C: STLV control cells without dextran; arrow points to microvilli in cross section.
Metabolism and lysis. The experiments with bead variations were conducted for only 2 days without feeding. At the conclusion of these experiments, glucose ranged from 130 to 250 mg/dl and pH from 7.2 to 7.6. Within each set of experiments (that is, different conditions run at the same time), glucose concentration varied by \(<15\%\). We also measured LDH activity in cell-free culture medium at the conclusion of the 2-day runs. Other researchers (13, 26, 29) have used this assay as a measure of cell lysis. We recorded LDH values in enzyme units per milliliter by comparison with a standard curve. Because basal levels in the inoculum culture varied, however, we report LDH values normalized by the average for a set of vessels started with the same inoculum (Fig. 2). The higher LDH level for stationary bag cultures was not statistically significant.

The experiments with medium variations, with or without dextran, were conducted for 5 days with identical feeding schedules. The glucose consumption showed almost identical patterns for dextran-treated and control vessels, and the pH was \(\approx7.0\) throughout.

Comparison of protein expression. VDR, specifically the membrane-bound fraction, was one of the proteins tested for possible variation between experimental conditions. Figure 3 demonstrates with antibody binding curves the relative levels of VDR for renal cell cultures in the STLV with the only difference between vessels being the size and/or density of the microcarrier beads. Replicate experiments were conducted on selected experimental conditions as noted, for the 1.03-sg small, 1.04-sg medium, and 1.04-sg large beads, to measure culture properties within a range of shear environments. Figure 4 shows the peak VDR antibody binding for these conditions. For cells attached to 1.04-sg large beads, binding was lower, at \(80\%\) compared with means of \(\approx110\%\) for the other STLV conditions. Analysis using KS statistics reveals that the bag cul-

![Fig. 2. Activity of lactate dehydrogenase (LDH) in cell-free medium at the end of the cultivation period for STLV and bag cultures; n = 8, 8, 4, 4, 3, and 3, respectively. Units of enzyme activity have been converted to percentages weighted by the average activity for a batch of experiments performed on the same day. There is no statistical significance between means by single-factor ANOVA when comparing each data pair in the group of glass-coated beads nor when comparing the final data pair in which Cytodex beads were used with or without dextran in the medium.](http://jap.physiology.org/)

![Fig. 3. Average vitamin D receptor (VDR) antibody binding curves for renal cells attached to beads in the STLV and bag. The membrane-bound fraction of VDR was isolated, and indirect fluorescent antibody binding was assayed by flow cytometry. Fluorescent units of antibody binding are shown vs. antibody dilution on a log scale. Solid curves represent the following samples with replication number as indicated: STLV with 1.03-sg small glass-coated beads (■), \(n = 5\); STLV with 1.04-sg medium beads (○), \(n = 6\); STLV with 1.04-sg large beads (▲), \(n = 4\); and stationary bag with an equal mixture of these 3 types of beads (△), \(n = 2\). Shown also as dotted-line curves are 2 additional samples with \(n = 1\) each: STLV with 1.02-specific gravity (sg) glass-coated bead of average diameter 120 μm (●) and STLV with 1.04-sg bead of average diameter 120 μm (●).](http://jap.physiology.org/)
ture had significantly lower antibody binding compared with the STLV cultures.

Also shown in Fig. 4 are the peak VDR levels for another set of experiments in which the medium density was changed by the addition of dextran. With dextran, the fluid density approaches that of the microcarrier beads, but fluid viscosity is increased, resulting in a fairly minor change in shear stress. By KS statistical analysis, VDR antibody binding to renal membranes was significantly lower in the dextran-treated group.

Another membrane protein measured in our experiments was DPP-IV. Although detected by flow cytometry, it was assayed functionally instead of the antibody binding being used to measure VDR. Figure 5 shows the fairly constant activity level of this protein over all the experimental conditions. No statistical difference was found.

The third protein measured in our experiments was cathepsin C, or dipeptidyl-peptidase I. Like DPP-IV, it was measured with a functional assay and detected by flow cytometry. Figure 6 demonstrates that, for one experimental condition, the cathepsin C activity was always significantly lower, when using either KS statistics or single-factor ANOVA. This condition was cells in the STLV attached to 1.04-sg medium beads.

Total protein was measured on the cellular membrane fraction for each sample isolated for the specific protein assays. Those results (Fig. 7) demonstrate that total protein was statistically similar except for the STLV with 1.04-sg medium beads.

**Correlation with shear.** By using estimates of shear stress, as presented in Table 1, the relative concentration or enzyme activity of the three measured proteins was plotted vs. shear to examine the relative relationships. Figure 8 shows three very different shear dependencies for measurements of these three proteins. In the following section, we will discuss the potential biological implications of this result.
DISCUSSION

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 (1, 17). Suspension culture continues to be useful for production of many bioproducts, from antibodies to hormones (1, 35). 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. Engineering optimization of suspension culture was largely undertaken by National Aeronautics and Space Administration engineers to model culture conditions in spaceflight (13, 22, 37) but may find its greatest utility in the carryover to ground-based applications (13, 16, 35).

Engineering optimization of the RWV to minimize shear while maintaining laminar flow has been successfully modeled and experimentally validated (10, 11, 13, 22, 27, 32, 37). However, the engineering is so effective that residual shear in the vessel is likely to be far below the in vivo levels experienced by many tissues such as vascular endothelium, renal proximal tubules, and blood cells. One interesting question is how this environment will affect the cultured cell’s biological behavior in terms of protein expression.

RWV culture provides a complex set of dynamic culture conditions, in which the quantitative importance of each physical parameter to the observed biological effects remains unknown. Understanding the effects of the RWV has commonly been approached by comparing cells grown in the vessel to diverse control conditions ranging from conventional two-dimensional monolayer cultures, through stirred fermentors, and nonadherent culture bags (13, 16, 17, 35). To begin to dissect the mechanistic issues, we chose to change a single parameter (medium density, bead size, or bead density) in comparing cell behavior in a set of otherwise identical RWVs. This approach begins to bring these experiments into a much more controlled and interpretable setting, with defined variables to modify and observe.

Terminal velocity and shear stress in the RWV. The RWV suspends cells by rotation, which introduces centrifugal and Coriolis pseudoforces. A particle in the RWV traces a small circular path while it is rotating with the fluid in a much larger circular path. A force balance on the particle shows that in the radial direction centrifugal force and gravity counteract each other, whereas in the tangential direction gravity and Coriolis force are additive. The particle’s velocity components are functions of its $V_t$ and the rotational speed of the vessel (37). The terminal or sedimentation velocity ($V_t$) is defined by Eq. 1 in MATERIALS AND METHODS as the equilibrium velocity at which a particle moves under the influence of gravity. Equation 1 suggests methods of reducing $V_t$ under unit gravity; for example, driving the density of particle and fluid closer together, decreasing particle size, or increasing fluid viscosity. When the vessel is operated at low rotational speeds, the gravitational effect dominates and parti-

![Fig. 7. Average total membrane protein concentration for STLV and bag cultures; n = 6, 6, 4, and 2, respectively. Comparison between the first 2 data points shows that STLVs with the 1.04-sg medium beads (*) had higher levels of total membrane protein than STLVs with the 1.03-sg small beads, with $P < 0.03$. This finding does not affect any other results.](image)

![Fig. 8. Plots of enzyme activity or peak antibody binding related to shear stress associated with each experimental condition. Terminal velocities were calculated from Eq. 1 and maximum shear stress on a single cell-coated bead from Eq. 2 in METHODS. This yields not an absolute but a relative value of shear stress suitable for purposes of comparison between experiments. The 3 different renal-specific proteins tested show vastly different patterns of expression or activity with shear, indicating that these effects are specific. Polynomial curves or lines were included in the figure to show the approximate trends in protein expression with shear for VDR (●), DPP-IV (○), and cathepsin C (■).](image)
icles tend to settle. When the vessel is operated at high rotational speeds, centrifugal effects dominate, accumulating with time, so that particles move out toward the wall of the vessel. Typically, aggregate size will increase as cells grow over time in the vessel. Thus rotational speed may need to be adjusted to promote ideal suspension conditions and avoid settling or excessive wall impacts.

Equation 2 in MATERIALS AND METHODS describes the maximum shear stress ($\tau_{max}$) as a function of the $V_t$. Both Eqs. 1 and 2 apply to creeping flow around a solid sphere (2), which adequately describes the movement of a bead particle inside the RWV. This is true when the Reynolds number ($Re = 2\rho_V V_t / \mu$) is less than 0.1 (2), which is the case for all of our experimental conditions except for the largest beads, the 1.04-sg beads with an average diameter of 275 $\mu$m. For this condition, the Reynolds number is $\sim 0.3$. There are other expressions for estimating the $V_t$ for higher Reynolds numbers. However, for $Re < 2$, the present expression is more accurate (2). Hence, it is reasonable to apply the same equations to all of our experimental conditions.

Gao et al. (11), in their model of bead motion in the RWV, 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 $V_t$. They show the periodic nature of the relative velocity, and hence 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 ($\mu$), density difference ($\Delta \rho$) between particle and fluid, microcarrier bead radius ($R$), vessel rotational speed ($\omega$), and initial particle position ($\tau_0$). They found that the maximum shear stress increases linearly with particle radius and with the density difference. Maximum shear stress is constant, however, with respect to fluid viscosity and vessel speed. The authors suggest that the opposing effect of viscosity on relative velocity cancels its effect on shear stress. Although $\tau_{max}$ is not dependent on $\omega$ or $\mu$, another important parameter is, i.e., the time until wall impact, which varies inversely with $\omega$ and increases linearly with $\mu$. 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 RWV are density difference and particle radius, and these are indeed the variables that are permuted in the current work.

Validity of comparison. The engineering calculation of $V_t$ and shear is based on a simplified initial condition of cells coating single beads. As the culture matures and larger aggregates form, the $V_t$ and shear evolve dynamically. We have examined the outcome at the end of 2 days in culture. Future studies may need to follow the time course and/or quantify aggregate size over time for a more accurate assessment of experimental conditions in the vessel.

Because of the body of evidence that shear stress modifies gene and protein expression in vascular endothelial cells, shear stress is often quoted as a biologically active culture parameter in the RWV (13, 20, 22). To test the importance of shear in our system, we devised an initial set of experiments in which, by adding dextran to the vessel, we cut the $V_t$ of the cell aggregates by more than half but changed shear relatively little. Perhaps as significant is that wall impacts were theoretically reduced with the addition of dextran because the fluid viscosity increased. In this setting, there was no effect on the assay of VDR. This provides direct evidence that $V_t$ per se, and perhaps wall impacts as well, has little effect on at least one renal-specific protein. In addition, this provides indirect evidence that shear is important in the changes observed in subsequent experiments.

There was at least one physical difference between culture conditions, that of aggregate morphology. The variable that we could control was bead coverage, so that all cultures were inoculated with the same number of cells per square centimeter of bead surface. At the conclusion of the experiment, the number of beads per aggregate was less for the 1.04-sg large-bead condition, which could have affected the potential for cell-cell contacts. Interactions between cells are generally accepted as important for modulating control of the cell cycle and apoptosis (33). However, we found no evidence that the cell cycle, cell death, or lysis varied to any extent for the different conditions in this study. Given the large size of the beads in question, the reduced bead number does not necessarily correspond to smaller aggregate size. Again, it is aggregate diameter that affects $V_t$, shear, and wall impacts. Monitoring the dynamic changes in aggregate diameter would be a logical extension to this work.

The protein expression differences we observed are apparently not related to modulations in total protein. Total protein levels were similar between conditions, with the exception of higher protein in the STLV with 1.04-sg medium beads compared with the smaller diameter beads. However, lower levels of cathepsin C for the 1.04-sg medium-bead condition cannot be attributed to lower total protein. And, for the other two proteins studied, VDR and DPP-IV, levels were not higher solely for this vessel condition.

Many changes in mechanical culture conditions can also change mass transport (10, 32). However, unlike cells growing in the quiescent conditions of spaceflight (17, 32, 37), aggregates spinning in a rotating vessel have adequate mass transport because of bulk flow (5, 11, 35). Similarly, it is unlikely that the observed changes are nonspecific toxic effects because there was no effect on cell cycle, cell death, or lysis. This is particularly important given evidence of enhanced cell death by apoptosis and necrosis for insect cells in a shaker flask, with slightly higher shear, compared with those in a RWV (5). Our results are consistent with mechanical forces mediating the observed changes.

Molecular markers. We chose to examine the biological effect of changing the mechanical parameters, $\Delta \rho$ and $R$, in terms of the expression of select proteins in...
our system. These were the VDR, and two aminopeptidases, cathepsin C (dipeptidyl-peptidase I) and DPP-IV. They were chosen on the following bases: the ability to measure these proteins by simple, reliable assay; their physiological importance as renal proteins; and previous demonstration of basal expression high enough to assay combined with changes in gene or protein expression observed in our laboratory’s earlier RWV studies (16, 20).

VDR binds the active form of the vitamin D hormone, 1,25-(OH)$_2$ vitamin D$_3$, to create a transcriptional complex. It is one of a family of nuclear receptors and, besides kidney, is present in other target tissues of vitamin D (19). Much is known about the DNA and ligand binding activities of this receptor but not about its potential associations with other proteins (19). A portion of the total VDR is partitioned to the membrane as we are able to measure specific binding from the membrane fraction of cultured renal cells. Cathepsin C is a predominantly lysosomal aminopeptidase that is widely distributed in mammalian tissues, but especially in the kidney and spleen (34). Its main function is protein degradation in the lysosomes. DPP-IV is a glycoprotein found on the brush border membrane of cells of the proximal tubule and glomerulus and is also found in liver, parotid, and salivary glands. It has a transmembrane segment, but 95% is extracellular. Among its myriad functions are proteolysis of bradykinin and substance P and binding to the extracellular matrix components collagen and fibronectin (36).

**Relating biological results to mechanical properties.** The pattern of changes in VDR, cathepsin C, and DPP-IV with changes in shear has instructive characteristics. First, although abundantly expressed, DPP-IV activity did not change with any modification in culture conditions. Because DPP-IV is an apical brush border membrane enzyme, this suggests that the other changes observed are highly specific changes and are not due simply to a nonspecific or general change in all protein production or membrane cycling. The rapid biological turnover of DPP-IV (21) suggests that these changes are valid and not simply secondary to a long membrane residence time of this marker. Second, there is a relationship between VDR expression and shear, such that optimal expression occurs at the midrange of dynamic shear conditions examined and not the lowest shear possible. Third, cathepsin C had an inverse relationship to shear, being suppressed at mid levels of shear. Hence, the pattern of change with modulation of shear is consistent and reproducible for each protein examined, but the protein changes demonstrated diverse patterns and specificity of response.

Although the specific signal transduction mechanisms from mechanical culture conditions to changes in gene and protein expression are incompletely understood, there are several important clues. First, there are precedents for other mechanical culture conditions changing gene expression (8, 9, 12, 13, 17, 25, 28, 30, 31, 39). In particular, heat shock proteins may transduce changes in temperature into molecular cell processes by heat-dependent conformational changes in chaperone proteins (7, 24, 39). Similarly, both vibration and gravity mediate specific gene responses in osteoblasts and Jurkat cells, including but not limited to changes in heat shock proteins and c-Fos (8, 9, 18, 23, 24). This increase in c-Fos is cAMP but not protein kinase C dependent (9). Several lines of evidence, including the identification of transcriptional binding sites and candidate transcription factors, suggest that shear stress can mediate select changes in cellular gene expression in vascular endothelial cells (12, 24, 28, 30). The central role of the cytoskeleton in cell scaffolding, load bearing, and transport of vesicles continues to make it a popular candidate for transducing physical stimuli into other cellular processes (23, 24, 32). 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 spaceflown human lymphocytes (23, 24).

There is some uncertainty as to the actual level of shear experienced by the renal cell. A recent study by Guo et al. (14) models the response of renal cells to flow inside the proximal tubule. Their model shows that, although shear stress varies from 1 to 5 dyn/cm$^2$ over the normal range of tubule flow rates, shear at the base of the microvilli is several hundred times smaller. That is, most of the drag force from fluid flow affects only the tip of the microvillus. In our own experimental system, the cells may not have the same structural organization as in the proximal tubule. Therefore, we must relate the observed biological differences in our experiments to relative values of shear stress.

This study provides direct evidence that mechanical culture conditions modulate protein expression in suspension culture. When shear and $V_t$ are dissociated by changing the fluid viscosity, $V_t$ alone has no effect on protein expression or activity. The responses of protein expression or activity to changes in the shear environment were highly specific for each protein. Future studies may examine the effect on other proteins important to renal cell culture.

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