Characterization of aggregation and protein expression of bovine corneal endothelial cells as microcarrier cultures in a rotating-wall vessel

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Received 13 January 2000; accepted 13 January 2000

Key words: aggregation, bovine corneal endothelial (BCE) cells, kinetic model, microcarrier beads, protein expression, rotating-wall vessel (RWV)

Abstract

Rotating-wall vessels are beneficial to tissue engineering in that the reconstituted tissue formed in these low-shear bioreactors undergoes extensive three-dimensional growth and differentiation. In the present study, bovine corneal endothelial (BCE) cells were grown in a high-aspect rotating-wall vessel (HARV) attached to collagen-coated Cytodex-3 beads as a representative monolayer culture to investigate factors during HARV cultivation which affect three-dimensional growth and protein expression. A collagen type I substratum in T-flask control cultures increased cell density of BCE cells at confluence by 40% and altered the expression of select proteins (43, 50 and 210 kDa). The low-shear environment in the HARV facilitated cell bridging between microcarrier beads to form aggregates containing upwards of 23 beads each, but it did not promote multilayer growth. A kinetic model of microcarrier aggregation was developed which indicates that the rate of aggregation between a single bead and an aggregate was nearly 10 times faster than between two aggregate and 60 times faster than between two single beads. These differences reflect changes in collision frequency and cell bridge formation. HARV cultivation altered the expression of cellular proteins (43 and 70 kDa) and matrix proteins (50, 73, 89 and 210 kDa) relative to controls perhaps due to hypoxia, fluid flow or distortion of cell shape. In addition to the insight that this work has provided into rotating-wall vessels, it could be useful in modeling aggregation in other cell systems, propagating human corneal endothelial cells for eye surgery and examining the response of endothelial cells to reduced shear.

Abbreviations: BCE, bovine corneal endothelial; HARV, high-aspect rotating-wall vessel; k_{11} , rate constant for bead-bead binding [day^{-1}]; k_{1A} , rate constant for bead-aggregate binding [day^{-1}]; k_{AA} , rate constant for aggregate-aggregate binding [day^{-1}]; n_1 , dimensionless number of single beads; n_A : dimensionless number of aggregates; N_1 , number of single beads; N_A , number of aggregates; N_0 , initial bead number; RWV, rotating-wall vessel; S , mean aggregate size; t , time.

Introduction

Reconstituted tissue from cell culture is designed to mimic the structural architecture and differentiated function of native tissue. These three-dimensional

(3D) cultures have application to *in vitro* drug testing where they have been, for over a decade, a desirable alternative to cell monolayers and organ cultures as a result of their *in vivo*-like response and availability (O'Connor, 1999). More recently, 3D cultures have

been useful in gene therapy development to assess, for example, gene penetration within tissue (Cheng et al., 1996). Furthermore, reconstituted tissue is beginning to replace the limited supply of donor organs for transplantation (Hansbrough et al., 1992).

Recent advances in culture technology have greatly improved the fidelity of reconstituted tissue. One such advance was the development of rotating-wall vessels (RWVs) by NASA (Schwarz et al., 1992). These bench-scale bioreactors were designed to simulate the cultivation of buoyant animal cells in microgravity by suspending cells in medium with less hydrodynamic forces than in conventional bioreactors. This is achieved by rotating the RWV around a horizontal axis for mixing, aerating the medium via a silicon membrane and filling the culture chamber completely with medium. The absence of an impeller, an air-liquid interface and shear at the chamber wall reduces shear stress in the vessel to approximately 0.2 dyne/cm^2 (Tsao et al., 1994). This quiescent culturing environment promotes 3D cell growth into multicellular aggregates and co-location of dissimilar cell types (Spaulding et al., 1993).

The RWV has proven beneficial not only to microgravity sciences but also to the field of tissue engineering. The reconstituted tissue formed in the RWV is characterized by more extensive 3D growth, tissue differentiation, and cell-cell and cell-matrix interactions than can be achieved in conventional bioreactors. For example, multicellular aggregates of human prostate tumor cells were more differentiated in terms of morphology and cytokeratin expression when grown in a RWV than control cultures in a spinner flask or static Transwell (Clejan et al., 1996). Likewise, reconstituted cartilage prepared from RWV cultures of chondrocytes had a higher content of matrix glycosaminoglycans relative to spinner-flask controls (Freed and Vunjak-Novakovic, 1995). As described in a review by Unsworth and Lelkes (1998), similar results were obtained for other types of tumor cells and primary cultures.

In the present study, we begin to investigate the mechanisms by which RWV cultivation affects the fidelity of reconstituted tissue. Of interest is the extent to which morphological and physiological properties of RWV cultures can be attributed to 3D cell growth or other aspects of cultivation. Bovine corneal endothelial (BCE) cells were selected as a representative monolayer culture for this study. They form a single cell layer *in vivo* on the posterior surface of the cornea and are attached to Descemet's membrane, a dense

extracellular matrix which separates the endothelium from the remainder of the cornea (Tuft and Coster, 1990). Our approach is to determine whether BCE cells exhibit aggregation and changes in protein expression in the RWV akin to those reported in the literature for cell lines that undergo 3D cell growth. In addition, the RWV could be useful in generating tissue for the repair of corneal endothelial deficiencies (Diaz-Valle et al., 1998) and in examining the response of endothelial cells to reduced shear (O'Neill, 1995).

Materials and methods

Cell cultivation

Primary cultures of BCE cells were established from whole calf eyes obtained at a local slaughter house (Crawford Meats, Franklinton, LA, USA) as previously performed in our laboratory (Blake and McLean, 1990). On the day of slaughter, the eyes were freed of adhering tissue with cool water, briefly sprayed with 70% EtOH, rinsed with cool distilled water, and covered for 15 min with gauze wetted with an aqueous disinfectant solution of $50 \mu\text{g/ml}$ chlorotetracycline (Sigma, St. Louis, MO, USA). After excising the cornea from eyes with a scalpel, endothelial cells were harvested and subcultured as described by MacCallum et al. (1982). Cultures of BCE cells were incubated at 37°C , 5% CO_2 and 95% relative humidity. The cells were maintained on T-flasks in GTSF-2 medium at pH 7.6 (Lelkes et al., 1997), containing 7% fetal calf serum (Gibco, Grand Island, NY, USA) and supplemented with an additional 2 mM glutamine (Sigma) immediately before use.

The RWV selected for this study was a 50 ml high-aspect rotating-wall vessel (HARV) manufactured by Synthecon (Houston, TX, USA). This particular class of RWVs has a high surface-area-to-volume ratio of 1.6 cm^{-1} to promote O_2/CO_2 exchange. In the HARV, BCE cells were grown in suspension on Cytodex-3 microcarrier beads (Sigma) at a concentration of $6.6 \times 10^3 \text{ bead ml}^{-1}$ with an average bead diameter of $175 \mu\text{m}$. The vessel was filled with 50 ml GTSF-2 medium and rotated at 25 rpm. Static T-flask controls contained 5 ml medium. In addition, 25 cm^2 T-flasks were each coated with 1.5 mg denatured porcine-skin collagen type I (Sigma) from an autoclaved 2% (w/v) stock solution. HARV cultures and T-flask controls were inoculated with tertiary BCE cells at a concentration of $3.3 \times 10^4 \text{ cells ml}^{-1}$. Both cultures were

fed with GTSF-2 starting two days after inoculation according to the following schedule: 10% of medium replenished on day 2 and two 20% refeeds each day thereafter. This feeding schedule kept the pH between 7.2 and 7.6 and glucose concentration between 80 and 135 mg dl⁻¹.

Glucose and cell concentration assays

Glucose concentrations were measured with a Yellow Springs Instrument Model 2700 Select Analyzer (YSI glucose membrane kit 2365, Yellow Springs, OH, USA). Cell concentrations were calculated from DNA content with Hoechst 33258 (Sigma), a fluorescent DNA-binding dye (West et al., 1985). Fluorescence was measured in a Luminescence LS50B Spectrometer (Perkin Elmer, Norwalk, CT, USA) with the excitation and emission wavelength set to 350 and 455 nm, respectively. The DNA standards for these measurements were prepared from salmon testes (Sigma). Fluorescence was converted to cell concentration from a calibration curve established from known concentrations of trypsinized BCE cells.

Scanning electron microscopy

Cell samples were prepared for analysis by scanning electron microscopy based on a procedure described by Goodwin et al. (1992). One-milliliter samples were first washed with PBS and then fixed in PBS containing 3% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde at pH 7.4 for a minimum of 4 hr. After three PBS washes (5 min each), the samples were postfixing with 1% (w/v) osmium tetroxide (Ted Pella Inc., Redding, CA) in PBS for one hour followed by three washes with distilled water. To quench postfixing and prevent microcarrier beads from collapsing, samples were suspended in 1% (w/v) thio-carbohydrazide (Electron Microscopy Sciences, Fort Washington, PA, USA) in PBS for 10 min and washed five times with distilled water. The samples were dehydrated with increasing concentrations of EtOH: 30, 50, 70, 95 and 100% for 5 min each followed by a second 5 min exposure to 100% EtOH. Once dehydrated, samples were dried with an Autosamdri-814 CO₂ critical point drier (Tousimis Research Corp., Rockville, MD, USA) and gold sputtered with an E-5000 Sputter Coater (Polaron Instruments, Hatfield, PA, USA). Processed samples were examined on a JSM-820 scanning electron microscope (Joel USA, Peabody, MA, USA).

Aggregate size determination and kinetic model

Aggregate size was determined with light microscopy from upwards of 150 aggregates in 30 to 50 randomly selected fields of vision. The mean aggregate size was the same whether the culture sample was removed from the HARV sample port or directly from the culture chamber. The coupled differential equations in our kinetic model of aggregation were solved by numerical integration using the Euler method with a time step of 0.01 days (Chan Man Fong et al., 1997). Kinetic constants were determined by least-squares fitting of predicted model results to experimental data. The merit function to be minimized was the sum of the squared residual error between predicted and actual values of aggregate size.

Protein radiolabeling and harvesting

Four days after inoculation, BCE cells were radiolabeled in conditioned GTSF-2 medium for 18 hr at 37 °C within the HARV and T-flask by adding 0.2 Mbq ml⁻¹ Tran ³⁵S-Label (ICN, Irvine, CA, USA) to the vessels during refeeding. Labeled proteins were harvested in the presence of protease inhibitors and separated into cell and extracellular matrix fractions as previously performed in our laboratory (Blake and McLean, 1990). Protein fractions were concentrated via centrifugation with a Amicon Centriplus-10 (Amicon, Beverly, MA, USA) spun at 3000 rpm and 4 °C for upwards of 4 hr. The amount of TCA-precipitable radioactivity in the fractions was measured on a Beckman LS 6000SC scintillation counter (Fullerton, CA, USA) according to Hugler et al. (1995). Between 45% and 94% of sample cpm was recovered after Amicon concentration.

Gel electrophoresis and autoradiography

Radiolabeled proteins in cell and matrix fractions were separated on a 7% (w/v) polyacrylamide gel containing sodium dodecyl sulfate at 200 volts with a Mini-Protean II Cell attached to a Model 500/1000 power supply (Bio-Rad, Richmond, CA, USA) as described by Laemmli (1970). Protein markers were ¹⁴C-methylated and had molecular weights in the range of 14.2 kDa to 205 kDa (Sigma). After electrophoresis, gels were dried at 80°C on a Model 543 vacuum dryer (Bio-Rad) and then submitted to autoradiography with X-OMAT AR scientific imaging film (Kodak, Rochester, NY, USA). Protein band intensities were measured with a Bio-Imaging Analyzer 1000

(Fuji Medical Systems, Stamford, CT, USA). These measurements were linear when 2×10^3 to 4×10^3 cpm were loaded per lane.

Statistical analysis

Significant differences in data were detected with analysis of variance followed by a Tukey test using SigmaStat, version 2.0 (Jandel, Chicago, IL, USA). A probability of less than or equal to 0.05 was the criterion of significance. All values are reported as mean $\pm$ standard deviation for at least duplicate cultures.

Results and discussion

Description of HARV culture and controls

For the majority of research with the HARV and other types of RWVs, attachment-dependent mammalian cells were grown in the bioreactor as a suspension culture bound to Cytodex-3 microcarrier beads (Unsworth and Lelkes, 1998). The same procedure was used herein to facilitate comparison of results. Also, there have been numerous studies of shear effects on microcarrier cultures of endothelial cells (O'Neill, 1995). The beads provide a high surface-area-to-volume ratio to grow large numbers of endothelial cells without the use of proteolytic enzymes which can be detrimental to fragile primary and early passaged cells (Ryan et al., 1980).

The control culture chambers for RWVs have been, with few exceptions, a plastic T-flask or petri dish (Unsworth and Lelkes, 1998). When bound to Cytodex-3 beads, cells attach to a layer of denatured porcine-skin collagen type I on the bead surface. Extracellular matrix proteins like collagen are known regulators of cell function (Davenport and Nettesheim, 1996). Since collagen type I is one of the matrix proteins synthesized by BCE cells and deposited into Descemet's membrane (Tseng et al., 1981), it could affect growth and protein expression in our system. As such, a more appropriate control for RWVs may be microcarrier beads in static culture or a T-flask coated with $60 \mu\text{g}/\text{cm}^2$ collagen type I, the concentration on Cytodex-3 beads. We selected the latter to magnify potential differences in protein synthesis between the HARV and control due to changes in cell shape from surface curvature. This study includes both collagen-coated and uncoated T-flasks as controls.

Figure 1. Growth profiles for BCE cells cultivated in a 50 ml HARV ($\square$), collagen-coated 25 cm^2 T-flask ($\blacktriangle$, $\blacksquare$) and non-coated 25 cm^2 T-flask ($\triangle$). The cultures were inoculated at either 7.0×10^3 cells cm^{-2} ($\blacksquare$, $\triangle$) or 3.75×10^3 cells cm^{-2} ($\square$, $\blacktriangle$). In addition, the HARV contained 6.6×10^3 beads ml^{-1} of Cytodex-3 microcarriers.

Growth profiles

A 50 ml HARV was inoculated with 3.3×10^4 BCE cells ml^{-1} at a ratio of 5 cells per Cytodex-3 bead. This corresponds to an inoculum of 3.75×10^3 cells per square centimeter of bead surface (Figure 1). BCE cells are very fragile: only one-third of the inoculum survived during the first eight hours of cultivation. After this time, the cells proliferated with a doubling time characteristic of *in vitro* BCE cultures, 28 ± 3 hr (Gospodarowicz et al., 1981), and reached a maximum cell density of 2.5×10^4 cells cm^{-2} .

Two different T-flask inocula were evaluated as controls for HARV cultures. Collagen-coated T-flasks were inoculated with the same concentration as in the HARV based on either medium volume (3.3×10^4 cells ml^{-1}) or surface area (3.75×10^3 cells cm^{-2}). For 25 cm^2 T-flasks containing 5 ml medium, the former is equivalent to 7×10^3 cells cm^{-2} . T-flasks prepared with the more concentrated inoculum had a growth profile similar to that of the HARV and, as such, were selected as controls in subsequent experiments (Figure 1). The only difference between HARV and control cultures was a 2.3-fold larger maximum cell density in the collagen-coated T-flask due to incomplete cell coverage of microcarriers within bead aggregates as described further in the next sec-

tion. In contrast, T-flasks inoculated with only 3.75×10^3 cells cm^{-2} had a longer lag phase than HARV cultures, and these cultures remained subconfluent throughout cultivation with a final cell density that was nearly three times less than that achieved in the HARV. Previously, some investigators have inoculated the HARV based on the surface area of microcarrier beads (Molnar et al., 1997). In light of our findings, medium volume may be a more appropriate basis.

As shown in Figure 1, the presence of collagen type I in T-flasks inoculated with 7×10^3 cells cm^{-2} had no effect on cell growth rate but resulted in a modest increase in maximum cell density from 4.15×10^4 cells cm^{-2} to 5.85×10^4 cells cm^{-2} on average ($p < 0.05$). This is consistent with the literature and most likely reflects a greater adherence and closer cell packing of BCE cells to the collagen substratum (Gherardini et al., 1997).

Culture morphology

Microcarrier cultures grown in RWVs are characterized by extensive 3D cell growth that initiates at cell bridges between beads, extends from the bridges into open pockets between beads, and eventually engulfs the beads (Clejan et al., 1996). These previous studies utilized fibroblasts and/or epithelial cells which form multilayers *in vivo*. As such, it has not been possible to determine whether 3D growth is due to the cell type or cultivation environment within RWVs. We have addressed this issue by characterizing the morphology of BCE cultures in the HARV with scanning electron microscopy.

The micrographs in Figure 2 reveal that endothelial growth was arrested when microcarrier beads were covered with only a monolayer. At confluence, BCE cells had a cobblestone appearance that resembled the mosaic structure of the native corneal endothelium (Tuft and Coster, 1990). There was substantial bead aggregation in the HARV (Figure 3). At the onset of confluence on day 7, bead aggregates contained 12 beads on average. This value nearly doubled during the remaining week of experimentation. Bead aggregation was negligible in the absence of cells (data not shown). Scanning electron microscopy revealed that beads within aggregates were linked by cell bridges composed of only a few cells each (Figure 2).

These findings suggest that cell bridge formation is common to attachment-dependent mammalian cells which form multilayers *in vivo* and those, such as BCE cells, that do not. Cell bridging is most likely the re-

Figure 2. Scanning electron micrographs of HARV cultures of BCE cells on day 14. Microcarrier cultures aggregated to form large bead clusters (A) that were linked together by cell bridges (B). Other features include the hexagonal shape of BCE cells on confluent beads and arrest of cell growth upon confluence. Magnification and scale are given in each micrograph.

sult of the quiescent environment within RWVs. Two microcarrier beads moving through culture medium can remain in close proximity to each other for longer periods of time than in a more turbulent bioreactor. Goodwin et al. (1993) demonstrated that lowering shear stress in RWVs by less than a factor of two from $0.92 \text{ dyne cm}^{-2}$ to 0.5 dyne cm^{-2} greatly increased the number of cell bridges in microcarrier cultures of baby hamster kidney cells. RWVs establish a fluid dynamic environment which enables, through cell bridge formation, cells to exhibit their innate propensity for 3D cell growth.

Last, the maximum cell density of HARV cultures can be estimated from scanning electron micrographs. For 50 beads examined, there were on average 34.5

Figure 3. Comparison of experimental data of aggregate size in the HARV ($\square$) with predicted model results (—). Also plotted are expressions for aggregate size in the limit as $t \rightarrow 0$ ($S = k_{11}k_{1A}t^2/2 + k_{11}t + 1$, $\diamond\diamond\diamond\diamond$; $S = k_{11}t + 1$, $\blacklozenge\blacklozenge\blacklozenge$) and as $t \rightarrow (\infty)$ ($S = k_{AA}t + \text{constant}$, $----$).

cells per half bead surface (Figure 2). Similar values (35 to 40 cells per half bead) were reported for Cytodex-3 cultures of neonatal human corneal endothelial cells (Insler and Lopez, 1990). According to Sigma's manufacturing specifications, 10^6 Cytodex-3 microcarriers have $1.15 \times 10^3 \text{ cm}^2$ of surface area for cell attachment. As such, 34.5 cells per half bead in the HARV corresponds to $6.0 \times 10^4 \text{ cells cm}^{-2}$, the same maximum cell density as in the collagen-coated T-flask control ($5.85 \pm 0.45 \times 10^4 \text{ cells cm}^{-2}$) within experimental error (Figure 1). As reported in the previous section, the maximum cell density that was actually achieved in HARV cultures was more than two-fold less ($2.5 \pm 0.6 \times 10^4 \text{ cells cm}^{-2}$). This discrepancy is most likely the result of incomplete cell coverage of microcarriers within bead aggregates.

Kinetic model of microcarrier bead aggregation

Microcarrier beads are popular supports for suspension cultures of attachment-dependent mammalian cells for both commercial and research applications (Blasey et al., 1995; O'Neill, 1995). Existing models for microcarrier cultures predict the frequency and energy of bead-bead collisions (Cherry and Papoutsakis, 1986; Croughan et al., 1988) and growth of attachment-dependent cells on microcarrier beads (Cherry and Papoutsakis, 1989; Hawboldt et al., 1994). The present study adds to this body of work

a kinetic model of bead aggregation. Such a model could provide (i) a means to predict bead aggregation, (ii) insight into the mechanisms of bead aggregation and (iii) a quantitative comparison of aggregation from different cultures.

This section presents a model of microcarrier bead aggregation which is based on equations derived for atoms clustering on surfaces (Zinke-Allmang et al., 1992). The microcarrier culture was divided into two groups: aggregates and single beads. Aggregates of all sizes have been lumped into the first group in this initial version of the model. To facilitate analysis, equations were derived in terms of dimensionless numbers of single beads (n_1) and aggregates (n_A), which varied between zero and unity, instead of the actual numbers which were as large as 3.3×10^5 at inoculation (N_0).

The resulting model includes the following coupled differential equations:

$$\frac{dn_1}{dt} = -2k_{11}n_1^2 - k_{1A}n_1n_A \quad (1)$$

$$\frac{dn_A}{dt} = k_{11}n_1^2 - k_{AA}n_A^2 \quad (2)$$

where n_1 is equal to the number of single beads (N_1) divided by N_0 ; n_A equals N_A/N_0 where N_A is the number of aggregates; the k 's are rate constants; and t is time. In Equation 1, the loss of single beads over time (dn_1/dt) is caused by the aggregation of single beads to other beads and single beads to aggregates. The accumulation of aggregates over time (dn_A/dt) in the second equation is a balance between the formation of aggregates from single beads and their depletion as existing aggregates cluster.

Model terms n_1 and n_A were related to experimental data in Figure 3 of the mean size (S) of aggregates and single beads combined. In Equation 3, S is expressed as the number of beads per aggregate.

$$S = (n_1 + n_A)^{-1} \quad (3)$$

The three model equations were solved by numerical integration, followed by least-squares fitting of predicted to actual size data. Boundary conditions for this solution were $n_1 = 1$ and $n_A = 0$ at $t = 0$. The model results shown in Figure 3 represent the actual size data within experimental error, with few exceptions. The fit was not significantly improved by allowing large aggregates to dissociate into either smaller aggregates or single beads, or by permitting three or more aggregates and/or beads to cluster at any given time (data

Figure 4. Dependence of the merit function on kinetic rate constants. The merit function is the sum of the squared residual error between predicted and actual aggregate sizes. Data in graph A were calculated by adjusting k_{1A} and k_{AA} to minimize the merit function for given values of k_{11} . Likewise, the merit function was evaluated for values of k_{1A} in graph B and k_{AA} in graph C. The single and narrowly defined minimum at 0.14 day^{-1} for k_{11} , 0.97 day^{-1} for k_{AA} and 8.4 day^{-1} for k_{1A} indicates a unique solution to model equations.

not shown). The insensitivity of the model solution to these changes suggests that they are secondary to the dominant mechanisms of aggregation: bead-bead, bead-aggregate and aggregate-aggregate binding. Further, lumping all aggregates into one group may have obscured dissociation for a subset of aggregate sizes.

The kinetic rate constants in the model were adjusted to minimize the sum of the squared residual error between predicted and actual values of aggregate size. This merit function has a single and narrowly defined minimum at 0.14 day^{-1} for k_{11} , 0.97 day^{-1} for k_{AA} and 8.4 day^{-1} for k_{1A} , indicating a unique solution to the model equations (Figure 4). These best-fit rate constants indicate that the rate of aggregation between a single bead and an aggregate was nearly 10 times faster than between two aggregates and was 60 times faster than between two single beads. There was no significant improvement in model fit to experimental data when k_{AA} was permitted to be a function of aggregate size (data not shown).

The frequency of aggregation described by the kinetic rate constants reflects a composite of physical and biological events: bead collisions and cell bridge formation. If only bead collisions were considered, the rate constants should increase with aggregate size. The collision frequency between two spheres is proportional to the cross-sectional area swept out by the spheres and their relative velocity (Atkins, 1990). The latter can be estimated from the terminal velocity of a sphere falling through a fluid (Bird et al., 1960). Since Stokes' law applies to experimental conditions described herein, terminal velocity increases with the square of the sphere radius.

From a biological perspective, a greater collision frequency between two large aggregates is likely to be offset by a diminished probability of cell bridge formation resulting from these collisions. The mass, terminal velocity and hence kinetic energy of an aggregate increase with its size. The probability that a mammalian cell can form a bridge between two aggregates is probably inversely related to their relative velocity. If cell bridging is initiated, it may be disrupted as cells absorb the energy of the colliding aggregates. This agrees with a k_{AA} less than k_{1A} . Another aspect of cell bridge formation to consider is available contact area on the surface of an aggregate. The contact area is greater between two aggregates than between two beads. Moreover, a single bead binding in a recess on the surface of an aggregate may have the largest contact area of the three groups. Increasing contact area should favor cell bridge formation. The trend in the kinetic rate constants is consistent with the available contact area.

The time dependence of n_1 and n_A predicted by the model provides further insight into microcarrier aggregation in BCE cultures (Figure 5). During the first 3 days of cultivation in the HARV, the model sug-

Figure 5. Model results for the dimensionless bead numbers n_1 (solid curve) and n_A (dashed).

gests that there was a 10-fold reduction in the number of single beads. In fact, the initial rate of single-bead aggregation ($k_{11}N_0$) was 4.6×10^4 beads day^{-1} . By the onset of confluence on day 7, n_1 was negligible. The model further suggests that the number of aggregates reached a maximum after the first two days of cultivation when beads had dimerized (Figures 3 and 5). The value of n_A at the maximum was 9.6×10^{-2} , and the ratio n_1/n_A ($[k_{AA}/k_{11}]^{0.5}$) equaled 2.6. On day 14, n_A had decreased to 4.6×10^{-2} from aggregate clustering. There was the potential for further aggregation after day 14 since the theoretical minimum of n_A (N_0^{-1}) occurs when all beads have clumped into one aggregate and has the value of 3.0×10^{-6} for our study. In practice, however, the lower limit of n_A is likely to be greater than its theoretical minimum as hydrodynamic forces, collisions with the reactor and other aspects of the reactor environment restrict aggregate size.

By considering the form of the model equations as time approaches zero and infinity, it is possible to obtain estimates of the kinetic constants directly from experimental size data without numerical integration. As $t \rightarrow 0$,

$$\lim_{t \rightarrow 0} S = \frac{k_{11}k_{1A}t^2}{2} + k_{11}t + 1. \quad (4)$$

For very small times, $t^2 \ll t$, and Equation 4 reduces to

$$\lim_{t \rightarrow 0} S = k_{11}t + 1. \quad (5)$$

As $t \rightarrow \infty$,

$$\lim_{t \rightarrow \infty} S = k_{AA}t + C \quad (6)$$

where C is an integration constant. Fitting Equations 5 and 6 to experimental size data, and then repeating the procedure with Equation 4 provides estimates of all three k values. Figure 3 contains plots of Equations 4–6 for the rate constants determined by numerical integration.

Also, these limiting forms provide a better understanding of the full model solution. For example, Equations 4 and 6 demonstrate that the sigmoidal shape of the model solution for aggregate size (Figure 3) reflects a transition in S from having a second-order to first-order dependence on time as cultivation progresses.

Protein expression

Multicellular aggregates grown in RWVs generally have a different pattern of protein expression relative to monolayer control cultures (Jessup et al., 1997; Lelkes et al., 1998). This is associated with the greater degree of differentiation in aggregates and is due, in part, to increased cell-cell and cell-matrix interactions that accompany 3D cell growth. The absence of 3D cell growth in HARV cultures of BCE cells make them ideal candidates to investigate whether other aspects of HARV cultivation affect protein expression.

For this study, BCE cells were radiolabeled in the HARV and T-flask during an 18 hr incubation with precursor after 4 days of cultivation. Labeled proteins were extracted from cultures into cell and extracellular matrix fractions and separated by molecular weight with gel electrophoresis. Autoradiography (Figure 6) and densitometry (Figure 7) revealed statistically significant changes in protein expression induced by HARV cultivation and the presence of collagen in T-flask controls. Because of the long incubation period, these data reflect a combination of protein synthesis and degradation: newly labeled proteins were synthesized within cells and deposited into extracellular matrix as older labeled proteins were degraded by proteases during cellular turnover and matrix restructuring. As controls, Figures 6 and 7 include representative proteins (48 and 102 kDa) whose expression was unaffected by the culture variables under investigation.

Accumulation of a 210 kDa matrix protein was enhanced slightly by coating the plastic substratum in T-flasks with denatured collagen type I prior to cultivation. As shown in Figure 7, this protein accounted for $12.0 \pm 0.8\%$ of the total radiolabeled

Figure 6. Autoradiograms of ^{35}S -labeled proteins extracted from BCE cells and separated into cell (A) and extracellular matrix (B) fractions. BCE cultures were grown in the HARV (lane 1A/B), collagen-coated T-flask (2A/B) and non-coated T-flask (3A/B). Gel A was loaded with $3.5 \pm 0.1 \times 10^3$ cpm per lane; gel B, $2.5 \pm 0.5 \times 10^3$ cpm per lane. Protein markers (4A) were ^{14}C -methylated and ranged in size from 14.2 kDa to 205 kDa. An asterisk denotes control proteins whose expression was unaffected by the culture variables under investigation.

Figure 7. Densitometric analysis of autoradiograms in Figure 5. BCE cells were cultured in the HARV (■), collagen-coated T-flask (□) and non-coated T-flask (▨). Radiolabeled proteins were separated into cell (A) and matrix (B) fractions.

protein in the extracellular matrix of collagen-coated controls versus $7.9 \pm 1.9\%$ for uncoated controls ($p = 0.025$). The substratum is known to influence expression of extracellular matrix proteins. For example, deposition of fibronectin and laminin was evident under undifferentiated and differentiated cultures of primary rat tracheal epithelial cells grown atop a membrane coated with collagen type I (Davenport and Nettesheim, 1996). On uncoated membranes, however, these matrix proteins were not detectable for undifferentiated cultures and were limited to patches of fibronectin for differentiated cultures. It is note-

worthy that greater protein deposition was not caused by enhanced transcription. On the contrary, gene expression for matrix proteins was down-regulated in rat tracheal epithelial cells maintained on collagen-coated membranes. Both fibronectin and laminin (B chain) are expressed by BCE cells and have molecular weights similar to that of the 210 kDa protein in our study (Munjal et al., 1990).

Densitometry revealed that several proteins in the cellular fraction (43 and 70 kDa) and matrix fraction (50, 73, 89 and 210 kDa) were affected by HARV cultivation (Figures 6 and 7). The 70 kDa protein was highly enriched in HARV cultures. It accounted for 16% of total incorporated radioactivity in the cell fraction of HARV cultures which was 8 times the value for the two controls. Accumulation of the 43 kDa protein in the HARV was less than that of the collagen-coated T-flask by 30% on average ($p = 0.015$). For the matrix proteins, accumulation of both the 73 and 89 kDa proteins in HARV cultures was double the value for the controls. The converse was true of the 50 and 210 kDa matrix proteins.

For the 43 kDa cellular protein as well as the 50 and 210 kDa matrix proteins, collagen coating the T-flask significantly altered the relative densitometric results between the HARV and control (Figure 7). Consider the 43 and 50 kDa proteins. The HARV and collagen-coated control yielded statistically different results as described above, but the difference in band intensities between the HARV and uncoated control was not significant ($p > 0.05$). The effect that collagen had on protein expression was likely due in part to changes in cell shape resulting from closer cell packing on this matrix (Figure 1). It is well-documented that cell shape regulates protein expression by stimulating signal transduction from the cell membrane through the cytoskeleton and nuclear matrix to the genome (Ben-Ze'ev et al., 1980).

Our findings demonstrate that during HARV cultivation factors other than 3D cell growth can alter protein expression relative to that in the collagen-coated control. Perhaps there was a time lag in protein expression between the two cultures; however, this is unlikely since their growth profiles in Figure 1 are so similar. More likely, protein expression was affected by hypoxia, fluid flow and/or a distortion in cell shape in the HARV. Cells within large aggregates can become hypoxic due to reduced mass transport. Oxygen deprivation induces expression of a subset of stress proteins in animal cells, including one with a molecular weight of 70 kDa (Hugler et al., 1996).

Four days after inoculation, the average aggregate size was 6 beads per aggregate, and the synthesis of a 70 kDa cellular protein was up-regulated 8-fold. Fluid flow, at physiological levels, is an important stimulus for a variety of endothelial processes, including nitric oxide release (O'Neill, 1995). As such, the gentle mixing in the HARV relative to the static environment in the T-flask may have had a significant effect on protein expression in BCE cells. Last, the importance of cell shape in regulating protein expression was described above. Cells grown on microcarrier beads in the HARV relative to monolayer T-flask controls had a greater curvature and were stretched as bridges between beads. These distortions could have contributed to the differences in protein expression observed in the two culture vessels.

Conclusion

As a representative monolayer culture, BCE cells provided insight into factors during HARV cultivation which affect three-dimensional growth and protein expression. Specifically, the HARV and other RWVs establish a quiescent fluid dynamic environment which enables cells, through cell bridge formation, to exhibit their innate propensity for 3D growth. The collagen coating on Cytodex-3 beads aids in cell attachment to microcarriers; however, this matrix has secondary effects on cell physiology and metabolism, including cell density and protein expression. Last, HARV cultivation alters protein expression relative to collagen-coated T-flask controls in the absence of 3D growth perhaps as a result of hypoxia, fluid flow and/or a distortion of cell shape. This study should be useful in modeling aggregation in a variety of cell systems, propagating human corneal endothelial cells for eye surgery, and examining the response of endothelial cells in general to low levels of shear. Future research in our lab will incorporate multiple aggregate sizes into the kinetic model and expand the analysis of protein expression in HARV and control cultures.

Acknowledgments

This work was supported by grants from NASA (NAG9-826, KO), NIH (EY09092, DB) the Greater New Orleans Foundation (KO) and the Louisiana Space Consortium (KO). Mr. Robert Dotson, Ms. Zuzana Hruska and Ms. Karen Francis provided technical assistance with scanning electron microscopy.

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