Oxygen Is a Factor Determining In Vitro Tissue Assembly: Effects on Attachment and Spreading of Hepatocytes

Avi Rotem, Mehmet Toner, Sangeeta Bhatia, Brent D. Foy, and Ronald G. Tompkins, and
Surgical Services, Massachusetts General Hospital, and the Shriners Burns Institute, Department of Surgery, Harvard Medical School, Boston, Massachusetts 02114

Martin L. Yarmush*
Department of Chemical and Biochemical Engineering, Rutgers University, Piscataway, New Jersey 08855

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Many recent studies related to the development of bioartificial liver devices have utilized hepatocytes cultured within devices of various geometries. Because hepatocytes are anchorage-dependent cells, they need to attach and spread onto the extracellular matrix to be able to function, a process that requires energy. Thus, it is important to deliver enough oxygen to hepatocytes contained within bioartificial liver devices during the early phase of cellular organization while the cells interact with the extracellular matrix. In this study, we investigated the effect of oxygen on the attachment and spreading of hepatocytes. Increasing the gas phase oxygen from 0 to 160 mmHg resulted in an increase in the percentage of cells attaching from 43.0 ± 5.8% to 103.6 ± 29%, 1 h after seeding. In a similar manner, increasing the gas phase oxygen from 0 to 160 mmHg resulted in an increase of the projected surface area from 310 ± 35 to 827 ± 127 μm^2, 24 h after seeding. Furthermore, the partial pressure of oxygen at the cell level was estimated using a diffusion-reaction model. The model indicated that a cell surface oxygen partial pressure of 0.064 mmHg was required for the half-maximal (K_m^a) attachment of hepatocytes to collagen-based substrate. On the other hand, the K_m^s value of the spreading process was predicted to be 0.13 mmHg. The results of this study demonstrate the importance of oxygen during the initial stages of attachment and spreading of hepatocytes, and it has important implications in the design of hepatocyte-based bioartificial liver devices.
Key words: hepatocytes • oxygen • artificial liver • bioartificial organs

INTRODUCTION

Although both acute and chronic liver patients can be treated effectively by whole organ transplantation, there are only about 3000 donor livers available annually in the United States, while approximately 30,000 patients die from liver failure.[17] Hepatocyte-based bioartificial liver support devices can be viewed as an adjunct, or in some cases, alternative, to whole organ transplantation. The design of a successful bioartificial liver device will require the use of a large number of functioning cells in a small volume. Because isolated hepatocytes need to attach and spread in order to function properly,[9] a clear understanding of the initial stages of the attachment and spreading processes would help in determining conditions for optimal hepatic tissue assembly in a device.

Recently our laboratory has reported the oxygen requirements of hepatocytes during the initial spreading period on a single collagen layer.[11] During the initial phase of culture (12 h), the spreading rate of hepatocytes was associated with an elevated oxygen uptake rate. This oxygen uptake rate was ~40% higher than the value observed during the late stable phase of culture. This high oxygen requirement of hepatocytes during their interaction with the collagen substrate is especially critical because inadequate oxygen supply to cells at the early phase of tissue reconstruction may lead to severe hypoxia and cell death. In a more recent study, Foy et al.[6] showed that oxygen is an important factor in the attachment of primary hepatocytes to microcarriers. In this study, a cell surface oxygen partial pressure of 0.1 to 0.2 mmHg was estimated to be necessary for the attachment of a large fraction of hepatocytes to microcarriers. Given the increased oxygen utilization in attaching hepatocytes, we sought to investigate in more detail the effects of oxygen on the interaction of hepatocytes with simple surfaces.

The present study documents the effects of oxygen on the attachment and spreading of hepatocytes on hydrated type I collagen gel. Oxygen partial pressure in the gas phase was varied, and the attachment and spreading were quantified by direct counting and video microscopy, respectively. Our results indicated that the attachment and spreading of hepatocytes required cell surface oxygen partial pressures of 0.064 and 0.13 mmHg, respectively.

* To whom all correspondence should be addressed.

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MATERIAL AND METHODS

Isolation of Hepatocytes

Hepatocytes were isolated from adult female Lewis rats (Charles River Laboratories, Boston, MA), weighing 180 to 200 g, by a two-step collagenase perfusion technique as described by Seglen,[13] and modified by our group.[5] Typically, 200 to 300 million hepatocytes were obtained from a single isolation with a viability of 85% to 95% as determined by exclusion of trypan blue dye.

Culture of Hepatocytes

P-60 Petri dishes (Falcon, Lincoln Park, NJ) were coated with 1 mL of type I collagen solution prepared from Lewis rat tail tendons.[5] The collagen gel was prepared by mixing nine parts of 1.11 mg/mL of collagen solution with one part of 10× concentrated solution of Dulbecco's Modified Eagle's Medium (DMEM, Hazelton, Lenexa, KS) chilled on ice, mixed just prior to use, and allowed to gel for 1 h at 37°C. The DMEM medium was supplemented with 0.06 mg/mL proline, fetal bovine serum, insulin, glucagon, epidermal growth factor, and hydrocortisone.[5] Unless otherwise stated, 2×10^6 viable cells were suspended in 2 mL of the medium and evenly spread on the collagen gel, and incubated at 37°C and 10% CO_2 under different partial pressures of oxygen. The P-60 Petri dishes were incubated within P-150 Petri dishes (Falcon), which were modified to include an inlet at the center of the lid to permit continuous flow of a gas mixture to the space surrounding the P-60 Petri dishes containing the cell cultures. Various gas mixtures of O_2, 10% CO_2, and balance N_2 were utilized at a gas flow rate of 0.5 L/min.

Attachment of Hepatocytes

Approximately two million freshly isolated and purified by Percoll gradient cells were seeded in 2 mL of conditioned media on a single layer of collagen gel in a P-60 tissue culture dish. Media was conditioned for 30 min prior to seeding of cells by equilibration with gas mixtures of various compositions. Furthermore, the collagen-coated dishes were conditioned by perfusion with the appropriate test gas mixture. Last, the cell suspension was perfused with a test gas mixture after plating, while the dishes were shaken to prevent attachment prior to complete equilibration with the appropriate gas mixture. Time zero began when shaking was stopped. Specifically, 10 mL of media in a T-25 flask (Falcon) was prepared for each experimental condition where gas mixtures were composed of 0% (0), 4% (30.4), 10% (76), 21% (160), 75% (570), and 90% (684 mmHg) O_2, 10% CO_2, and balance N_2. Gas phase oxygen partial pressures, which were expressed as a percentage of 760 mmHg, were not corrected for the water vapor pressure given the very small error encumbered by this emission (<5%). Cells were spread evenly and incubated under the aforementioned has phase oxygen partial pressures. After 60 min, unattached cells were removed by immersion of a dish into a bath of Dulbecco's phosphate-buffered saline (PBS) at 37°C. For kinetic experiments, unattached cells were removed 5, 10, 20, 30, 45, or 60 min after seeding in environments of either 0 or 570 mmHg oxygen. The remaining, attached cells were incubated with 3 mL of 0.05% collagenase type IV (C-5138, Sigma), for 30 min at 37°C, and shaken at 100 rpm to create a cell suspension. The resuspended cells were collected and counted using a hemocytometer. Attachment was expressed in terms of the number of cells in suspension as a percentage of total number of cells seeded (i.e., $\sim 2 \times 10^6$ cells/dish). The process incurs a margin or error; therefore, on occasion, the mean value of detached cells exceeded 2×10^6 and the mean percentage of attached cells exceeded 100%.

Spreading of Hepatocytes

To study hepatocyte spreading, the partial pressure of oxygen that the cells were exposed to was varied using two methods. The first method used 30.4, 53.2, 76, 260, and 750 mmHg oxygen in the gas phase above the medium with a constant medium height of 1 mm. The media depth of 1 mm corresponds to 2 mL of media in a P-60 Petri dish. The second method used different media depths for a constant gas phase oxygen of 160 mmHg. For this second method, 2×10^6 cells were cultured in 2 mL of media and allowed to attach for 30 min. The media was then replaced with new medium at various heights of 0.5, 1.0, 1.5, 2.0, 3.0, 5.5, and 8.5 mm, and incubated for 24 h. For both methods, cells were cultured for 24 h, and then projected surface area was determined using a video microscopy system.[11]

Diffusion-Reaction Model for Oxygen Transport

To calculate the partial pressure of oxygen at the cell level, a one-dimensional diffusion-reaction model was used.[16] The solution of the diffusion-reaction model was given in Eq. (5) of Yarmush et al.[16] The following major assumptions were made in the model: (1) the mechanism of transport which dictates the oxygen transfer from the gas phase above the medium to the cells is by diffusion only; and (2) the oxygen uptake rate behavior of the cells follows Michaelis–Menten kinetics, in which the K_m^o is 0.5 mmHg oxygen as measured for suspended hepatocytes,[4,8] and the maximal oxygen uptake rate is 20 pmol/mg DNA/s as measured for hepatocytes several hours after seeding.[11]

Estimation of the Critical Diffusion Length

The diffusion-reaction model was also used to analyze the critical diffusion distance that resulted in oxygen limitation at the cell surface. The oxygen limitation for attachment of

spreading was defined as the condition for which the oxygen partial pressure at the cell surface was equal to the K_m^a or K_m^s value of hepatocytes. Using the diffusion-reaction model,[16] the relationship between the critical diffusion length and the gas phase oxygen partial pressure can be shown to yield:

$$\delta = \frac{Dk}{V_m \rho}\left[P_G\left(\frac{K_m^o}{P_C} + 1\right) - K_m^o - P_C\right] \quad (1)$$

where δ is the "critical" diffusion distance from bulk flow to the cells (cm), D is the diffusion constant (2×10^{-5}cm^2/s), V_m is the maximal specific oxygen uptake rate (0.4 nmol/s · 10^6 cells),[4] K_m^o is the oxygen partial pressure at the cell surface at which the oxygen uptake rate is half-maximal (0.5 mmHg),[4] ρ is the cell density (cells/cm^2), P_G is the gas phase partial pressure of oxygen (mmHg), P_C is the critical cell level partial pressure of oxygen and is equal to K_m^a (attachment) or K_m^s (spreading) (mmHg), and k is the solubility of oxygen in media (1.19 nmol/mL· mmHg).

Statistics and Data Analysis

Cell attachment experiments were repeated three times with two measurements in each experiment. For measurement of the projected surface area, experiments were repeated twice, five arbitrary fields were sampled in each experiment, and 7 to 15 cell per field were measured. All values were expressed as mean ± SD. Student's t-test was used for various statistical comparisons. To calculate the K_m values of the attachment and spreading, the results were fit by nonlinear regression to a curve generated assuming a Michaelis–Menten relationship.

RESULTS

Effect of Gas Phase Oxygen on Attachment

In order to determine the timing of attachment for dose experiments, hepatocytes were exposed to <1 and 570 mmHg gas phase oxygen partial pressures and their attachment kinetics were measured at different times following the seeding of hepatocytes onto the collagen gel (Fig. 1). In these experiments, the media depth and the cell seeding density were kept constant at 1 mm and 1×10^5 cell/cm^2, respectively. Thirty minutes after seeding, 96 ± 21% of the cells incubated under 570 mmHg oxygen were attached, as opposed to only 56 ± 13% of cells incubated <1 mmHg oxygen. The percent cells attached were significantly different at 30 min after seeding ($P < 0.05$). Increasing the culture time beyond 30 min did not cause an increase in the attachment of hepatocytes.

To determine the effect of oxygen on the attachment of hepatocytes, the oxygen partial pressure was varied between 30.4 and 684 mmHg gas phase oxygen. Given the fact that 30 min was enough for the attachment to reach saturation, we made our measurements 1 h after seeding to assure maximal attachment for a given oxygen condition. The media depth and the cell seeding density were kept constant at 1 mm and 1×10^5 cells/cm^2, respectively. Increasing

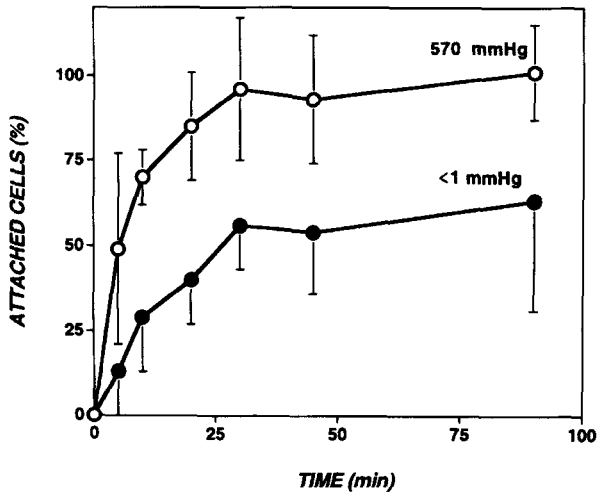

Figure 1. The effect of gas phase oxygen on the kinetics of hepatocyte attachment. Approximately 2×10^6 cells at a seeding density of 10^5 cells/cm^2 were placed onto collagen gel and incubated under gas phase oxygen of 0 and 570 mmHg. The media depth was 1 mm (i.e., 2 mL media in a P-60 culture dish). The results are an average ± SD of three experiments, two measurement for each experiment ($n = 6$).

the gas phase oxygen partial pressure from <1 to 76 mmHg caused an increase in the cell attachment from 43 ± 5.8% to 101 ± 20.7% (Fig. 2). Further increase in gas phase oxygen above 76 mmHg did not have significant effect on the attachment of hepatocytes to the collagen gel ($P > 0.05$). The hepatocyte viability did not decline during the 1 h (data not shown); therefore, it was unlikely that the reduction in attachment was a result of lower hepatocyte viability or dying cells.

Effect of Gas Phase Oxygen on Spreading

The effect of gas phase oxygen on spreading of hepatocytes onto the collagen gel was investigated using video

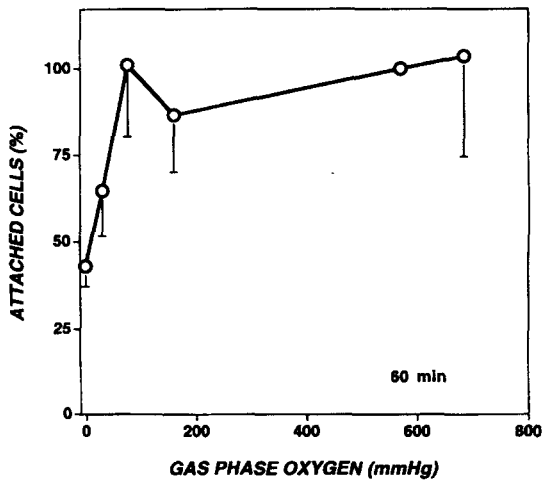

Figure 2. The effect of gas phase oxygen on cell attachment. Cells were seeded and incubated for 1 h under different gas phase oxygen. The results are an average ±SD of three experiments, two measurement for each experiment ($n = 6$).

microscopy. Throughout these experiments, media depth and cell seeding density were kept constant at 1 mm and 10^5 cells/cm^2, respectively. Cells were cultured under different gas phase oxygen partial pressures between 30.4 and 570 mmHg, and at different times the cultures were examined and the projected surface area recorded. Figure 3 shows the results of the kinetics of cell spreading onto a collagen gel layer during the first 24 h of culture after seeding. The projected surface area of cells incubated for 6 h on the collagen gel at low gas phase oxygen levels of 30.4, 53.2, and 76 mmHg displayed a maximum at 402 ± 51, 526 ± 60, and 552 ± 58 μm^2/cell, respectively. The projected surface area of hepatocytes incubated under high gas phase oxygen between 160 and 570 mmHg did not show a maximum, but rather increased steadily during the first 24 h to 640 ± 81 and 821 ± 150 μm^2/cell, respectively. The projected surface area after 24 h in culture as a function of the gas phase oxygen is plotted in Figure 4. As the oxygen increased from 30.4 to 570 mmHg, the projected surface area increased from approximately 310 to 810 μm^2/cell, respectively. The hepatocyte viability did not decrease during the 24-h exposure to oxygen concentration as low as 1%.

Analysis of the Cell Surface Oxygen for Attachment and Spreading

Oxygen from the gas phase was supplied to the monolayer of hepatocytes by diffusion through the media. Oxygen partial pressures at the cell level were estimated according to a one-dimensional diffusion-reaction model.[16] For example, at a gas phase oxygen of 30.4 mmHg, the cell level oxygen was 0.11 mmHg. Increasing the gas phase oxygen to 300 mmHg increased oxygen partial pressure at cell surface to 137 mmHg. These calculations were performed for a cell seeding density of 10^5 cells/cm^2.

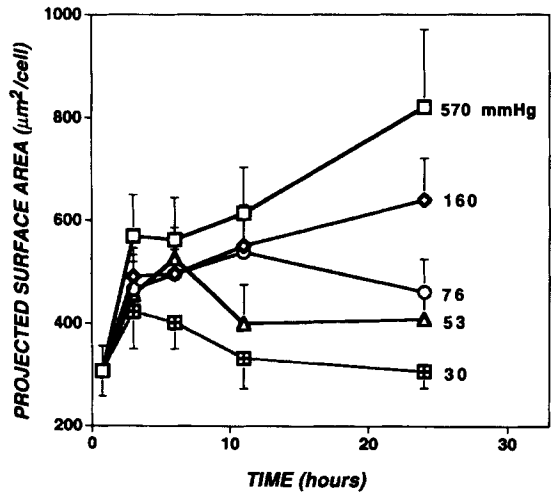

Figure 3. The effect of gas phase oxygen on the kinetics of hepatocyte spreading. Approximately 2 × 10^6 cells at a seeding density of 10^5 cells/cm^2 were placed onto collagen gel and incubated under various gas phase oxygen partial pressures. The media depth was 1 mm. The results are an average ± SD of five arbitrary fields and 7 to 15 cells/field for two sets of experiments (n = 80 to 100).

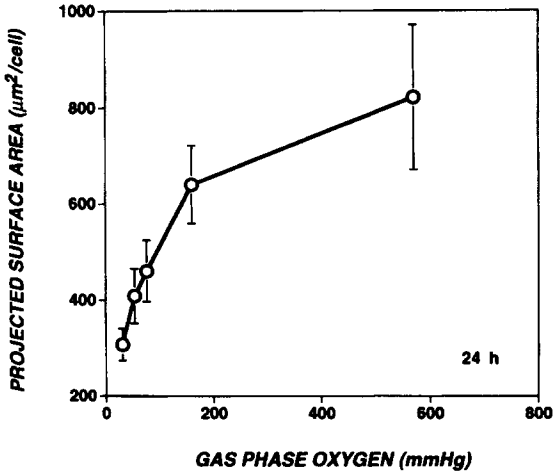

Figure 4. The effect of gas phase oxygen on cell spreading after a 24-h culture on collagen gel. Results were taken from Figure 3.

The gas phase oxygen data presented in Figures 2 and 4 were transformed using the diffusion-reaction model to obtain the dose response of attachment and spreading as a function of the oxygen partial pressure at cell surface. These results are depicted in Figure 5A and B. The K_m values for attachment and spreading were determined by fitting the data to a Michaelis–Menten relationship using nonlinear regression analysis. For attachment, a K_m^a value of 0.064 mmHg was obtained; whereas, for spreading, a K_m^s value of 0.13 mmHg was determined. Saturation occurred above 1 mmHg oxygen for both processes. The higher K_m^s value for spreading suggested that the spreading process was more sensitive to oxygen than the attachment.

The coordinate transformation from the gas phase oxygen (Figs. 2 and 4) to the cell surface partial pressure of oxygen (Fig. 5) required a number of approximations. To partially test the predictive ability of the model, and thus its underlying assumptions, experiments were performed by changing the media height at a constant gas phase oxygen of 160 mmHg. The media height was varied between 0.5 and 8.5 mm to create oxygen gradient in the media such that the partial pressure of oxygen at the cell surface was controlled. The oxygen at the cell level was estimated as described above, and the results are depicted in Figure 6. Using the Michaelis–Menten model, the K_m^s value of spreading when medium height was varied was determined to be 0.19 mmHg. Saturation for spreading occurred above 1 mmHg oxygen. These observations are very similar to those obtained by directly varying the gas phase oxygen at a constant media depth of 1 mm, supporting the use of the model to coordinate transfer from the gas phase oxygen to the cell surface oxygen.

Estimation of the Critical Diffusion Distance

In order to predict the critical diffusion distance (δ) above the cell layer (i.e., media height), at which the attachment

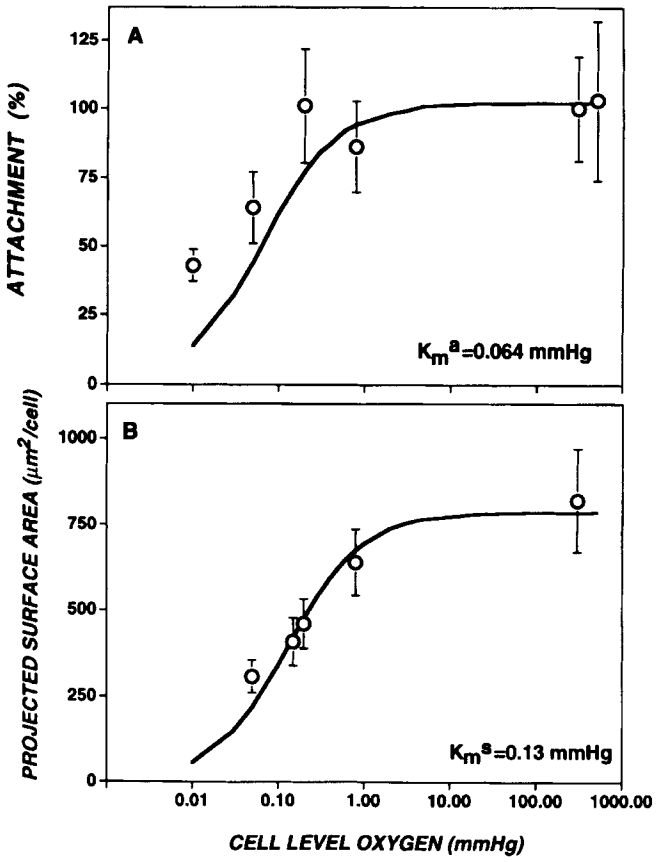

Figure 5. (A) Predicted K_m^a value for attachment. The data were taken from Figure 2. (B) Predicted K_m^s value for spreading when gas phase oxygen partial pressure was varied. The data were taken from Figure 4.

of hepatocytes was impaired, the diffusion-reaction model, Eq. (1), was used. In the calculations, it was assumed that the spreading was limiting as the oxygen partial pressure at the cell surface (P_C) was equal or below the K_m^s value for spreading (0.13 mmHg). Diffusion distances larger than δ were considered to be within an "oxygen-limited" zone. Figure 7 shows the critical diffusion length as a function of gas phase partial pressure of oxygen. As shown in Figure 7, the critical diffusion length increased as gas phase oxygen partial pressure increased and as cell seeding density decreased.

DISCUSSION

There are a number of different approaches to the development of hepatocyte-based bioartificial liver devices.[17] A common aspect of all these approaches is seeding of hepatocytes onto or within the biomaterial of choice. After isolation from intact liver, hepatocytes partially loose their phenotype and differentiated function. Upon seeding into a cell culture environment, they have to undergo an adaptation and reorganization process.[5,9] Initially, attachment and spreading onto the biomatrix must be performed in order to assume their normal geometry and function. In this work, we have shown that the efficiency of hepatocyte attachment and spreading strongly depends on oxygen

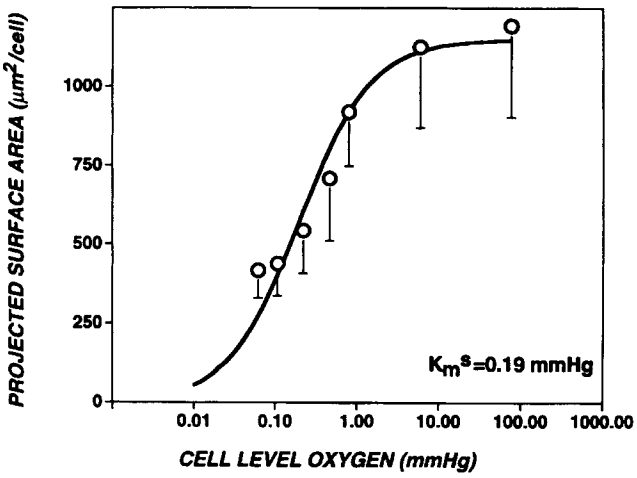

Figure 6. Predicted K_m^s value for spreading when media height was varied. Approximately 2×10^6 cells at a seeding density of 10^5 cells/cm^2 were placed onto collagen gel and incubated under 160 mmHg oxygen. The media depth was varied between 0.5 mm and 8.5 mm to achieve different oxygen partial pressures at the cell surface. The results are an average ± SD of five arbitrary fields and 7 to 15 cells/field for two sets of experiments (n = 80 to 100).

supply. Increasing the gas phase oxygen levels resulted in increased attachment and spreading.

Under low oxygen partial pressures in the gas phase (30.4 mmHg) the attachment was approximately half of that of cells cultured under high oxygen. This result suggests that attachment requires energy, which argues with the notion that attachment is a simple receptor–ligand reaction that does not require energy.[1,2] It is plausible that attachment is part of a complicated mechanism in which attachment and spreading are one continuous process, and as soon as the cell touches the substrate the spreading process begins. Thus, the low cell attachment (43%) achieved at 0 mmHg

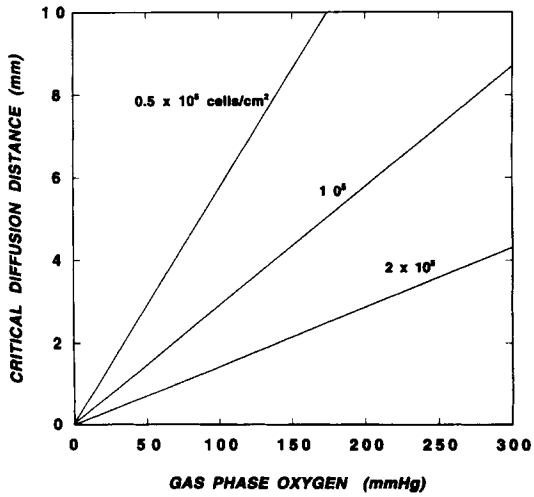

Figure 7. Predicted critical diffusion distance that results in impaired spreading as a function of gas phase oxygen partial pressure for three cell seeding densities. It was assumed that oxygen partial pressures at the cell surface (P_C) equal or less than the K_m^s (= 0.13 mmHg) value for spreading was limiting.

gas phase oxygen may represent pure attachment and corresponds to a receptor–ligand reaction only. The high cell attachment (100%) achieved at >160 mmHg gas phase oxygen would, therefore, be interpreted as a combination of both processes (i.e., attachment and the spreading).

Using a diffusion-reaction model, we estimated the cell surface oxygen pressure and determined that a critical cell surface oxygen partial pressure of 0.13 mmHg was required for the spreading of hepatocytes to collagen-based matrix. There are at least three parameters that influence the partial pressure of oxygen at the hepatocyte surface in the assembly of three-dimensional liver bioreactors: (1) the partial pressure of oxygen in the bulk fluid in the bioreactor; (2) the diffusion distance of the oxygen to hepatocytes; and (3) the cell seeding density. Although the values for these parameters depend on the exact type of the bioreactor, low partial pressures of oxygen within the bulk flow, long diffusion lengths, and high cell seeding densities will all reduce the oxygen partial pressure at cell surface which consequently limit cell attachment and spreading. Although the partial pressure of oxygen in the perfusate can be easily increased during the early phase of tissue assembly in vitro such as hollow-fiber devices, simple manipulations may not be possible in the case of transplantable cell–polymeric matrices. In this regard, and alternative approach, in which the transplantable materials are vascularized prior to the seeding of hepatocytes, has been suggested.[14]

Two previously published hepatocyte culture studies present data which can be qualitatively evaluated in terms of the oxygen effect on initial attachment and spreading. First, Cima et al.[3] have shown that under 160 mmHg oxygen, the attachment of hepatocytes to three different types of biomaterials (collagen and two polymer films) was independent of the surface type only for cell seeding densities less than 2×10^5 cells/cm^2. At higher cell seeding densities, a much greater number of cells attached to the collagen-coated substrates than to the polymer films. At 10^6 cells/cm^2, hepatocyte attachment was almost completely inhibited on all the substrates. One plausible explanation given in their study is that cell–cell interactions dominate the pattern of attachment at high seeding densities by promoting aggregate formation. However, using $K_m^a = 0.064$ mmHg and a cell seeding density of 10^6 cells/cm^2, the critical diffusion distance is 0.84 mm, much smaller than the media depth of 5 mm used in their study. Thus, it is possible that the oxygen limitation contributed to the observation made on the hepatocyte attachment process. Further work is clearly needed to better characterize the effects of oxygen on hepatocyte attachment on different surfaces. The differential attachment to various substrates at intermediate seeding densities may then be explained by the specific interaction of the substrate with hepatocytes as a function of oxygen tension. In a second study, the effect of oxygen on the albumin secretion of hepatocytes during the first day of culture was investigated.[16] When hepatocytes were plated at 10^5 cells/cm^2 under 160 mmHg gas phase oxygen, a media depth of 4 mm was detrimental to cell function and the observed albumin secretion rate was minimal. The critical diffusion distance for spreading estimated from Eq. (1) for these conditions was 4.6 mm. In addition, when the cell seeding density was increased twofold to 2×10^5 cells/cm^2 for 160 mmHg gas phase oxygen, the albumin secretion was compromised for media depths greater than 2 mm which is again in reasonable agreement with the theoretical prediction for δ of 2.3 mm. Thus, it is plausible that, in the absence of adequate oxygen, the hepatocytes cannot assume an appropriate physical state (i.e., by attaching and spreading) required for optimal albumin secretion.

In the in vitro reconstruction of hepatic tissue, an upper limit for the critical diffusion distance can be explored using the measured K_m^s value for hepatocyte spreading (Fig. 7). For example, in a flat-plate bioreactor using ambient oxygen at the inlet and a cell density of 2×10^5 cells/cm^2, the upper limit of the critical diffusion length is 2.3 mm. If there is an axial oxygen gradient in the bioreactor due to oxygen utilization by hepatocytes, cells that are at the downstream side of the bioreactor will require even shorter diffusion lengths. These calculations assume a monolayer of cells and a one-dimensional diffusion reaction model. In cases where there are multiple cell layers of aggregates, i.e., hollow-fiber bioreactors, the critical diffusion distance will be shorter. In a recent study, Nyberg et al.[10] and Shatford et al.[12] evaluated a contracted collagen hollow-fiber hepatocyte bioreactor in which the viability decreased radially with increasing depth inside the gel. It is plausible that the cells toward the inner region of the collagen lattice were never supplied with enough oxygen to properly interact with the collagen. One approach could be the use of high oxygen during the initial spreading phase, while hepatocytes have a ~40% increased a oxygen level of uptake rate.[11] This approach has recently been successfully used for attaching hepatocytes to collagen-coated microcarriers.[6] Alternatively, one might enhance oxygenation of the cells by shortening the diffusion distance between hepatocytes and perfusate.[7]

In summary, the results from this study indicate that oxygen plays a major role in the attachment and spreading of hepatocytes onto a collagen gel. As more and more complex three-dimensional hepatocyte bioreactor schemes are evaluated, this factor will loom prominent in their design.

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