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Improved oxygen diffusion and mechanical aggregation of tumor colonies in a novel stirred mini-bioreactor

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Abstract—**The feasibility of a novel stirred bioreactor, the rotating aerial disk (RAD) design, was tested in this study. The novelty lies in its method of medium recirculation by convective airflow using a non-contact planar disc, a variation on a physically defined theoretical model. Computational predictions of improved oxygenation were confirmed by increases in measured dissolved oxygen, even at Reynolds numbers (100-200) where flow is mostly laminar. EL-4 mouse lymphoma cells grown for the first time as suspension cultures in the RAD bioreactor, were mechanically re-organization into dense, circular three-dimensional colonies (diameter 3-5 mm, thickness 5-800 μm), more rapidly than we have observed previously. Cell proliferation in the RAD vessels was similar to static cultures, although lactate production from glucose was significantly lower, suggesting a shift toward aerobic glycolysis. This possible reversal of the 'Warburg effect' was accompanied by a decrease in mitochondrial activity, perhaps reflecting a more quiescent cytoplasmic state. The RAD device may be useful as scalable, three-dimensional solid tumor model under more physiological conditions than static culture.**

I. INTRODUCTION

Bioreactor technology is currently evolving toward the efficient expansion of human tissues, for a range of repair and regeneration strategies, often using progenitor cell types [1]. In this context, bioreactors can be regarded as fluid-driven long-term culture vessels that incorporate principles of fermentation technology and others forms of chemical engineering. Two important variables that have come under more scrutiny than others have included oxygen mass transfer and mechanical entrainment. Both parameters have become particularly relevant in cases when bioreactor environments are modified to contain surface coatings, synthetic scaffold materials and micro-carrier particles. While these biomaterials provide physical support to encourage three-dimensional tissue architecture, they also contain isolated microenvironments with localized stagnations in need of efficient oxygen. Induction of effective oxygenation delivery therefore remains very important in bioreactor design, whether stirred or perfused vessels.

While not continuously perfused, stirred vessels (eg. spinner flasks) remain a popular class of batch bioreactor, with systematic induction of homogenous nutrient distribution and controllable fluid mechanics. A major drawback of this and other classical stirred designs has been the variability of stress/strain imparted by irregularly shaped impellers. Our group has previously overcome this mechanical variability by replacing the axial bladed impeller with a flat rotating surface, resulting in a robust laminar flow with stable, localized turbulence at a range of rotational speeds [2]. The same design has recently been modified further for tissue culture, providing an alternative long term model of three-dimensional tumor colony formation [3]. The main novelty in the design for tissue culture lies in its ability to simultaneously and controllably deliver oxygen and mechanical stress/strain, in a non-cytotoxic manner. The RAD bioreactor represents a further optimization towards even smaller scale volumes, with geometries closer to static tissue cultures. By simple elimination of impeller immersion, this new variant also represents a safer culture system with potentially lower risk of contamination.

II. MATERIALS AND METHODS

A. Bioreactor design and culture conditions

The RAD culture vessel prototype was constructed using a standard six-well tissue culture plate (Falcon, BD Biosciences, St Louis, Mo, USA, Cat. No. 353502). The plate lid was replaced with a custom made polycarbonate support for six micro-stepper motors (RS components, Wetherill Park, NSW, Australia, Part No 535-0338) attached to a position encoder and motion controller (PXI, National Instruments, Austin TX, USA). Motors were mounted to the top surface of the support, with the axles protruding through drilled holes in alignment with the axes of each well. Pre-sterilized stainless-steel discs (diameter = 18 mm, width = 5 mm) were press-fit to the axles in close proximity to the inner surface of the lid (Fig. 1). Laminar flows of similar characteristics have been observed in physical models of a rotating disc in air, with robust characteristics even at very fast rotational speeds [4].

The RAD lid was used interchangeably for subsequent cultures of stirred (test) and static (control) cultures. Used discs and lids were dismantled, washed and autoclaved separately before replacement to new cultures. EL-4 aggregates were grown under similar conditions to those

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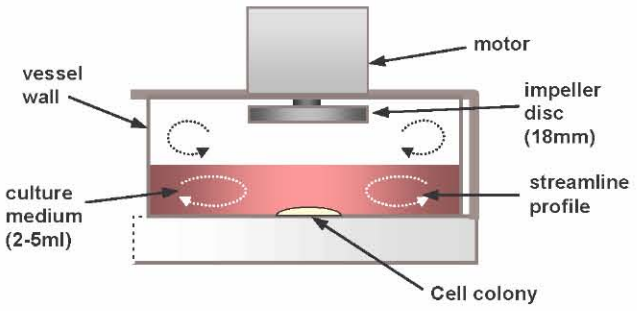

Fig. 1. Schematic of the rotating aerial disc (RAD) bioreactor

described previously [3], except for the 10-fold lower volumes of culture medium (Advanced Minimal Essential Medium (aMEM) supplemented with 10% Fetal Bovine Serum (FBS) Invitrogen, Carlsbad, Ca, USA). A fixed rotational speed of 120 rpm (Reynolds number ≈ 118, calculations based on outer edge speed of the disc, and cylinder radius) was chosen for this study, based on previous flow optimizations (not shown). The other exception is the presence of anticlockwise recirculation profiles (Fig. 1) opposite to those determined previously for a rotating apical disc [3] but similar to those of a rotating base configuration [2]. Overall, the bulk flow in transverse planes through the medium volume follows that of disc rotation.

B. Computational modelling of RAD cultures

Numerical modelling of fluid flow and oxygen transfer within the RAD vessel was performed using a commercial computational fluid dynamics (CFD) software package, FLUENT version 6.3 (Ansys, Lebanon, NH USA). The unsteady, axisymmetric and incompressible Navier-Stokes solver was used, in which the simulation was time-marched from zero flow initialisation to the asymptotic steady-state. Conditions at the air-medium boundary in between were solved using the Volume-of-Fluids approach. The dilute approximation that assumes constant fluid-phase density and oxygen diffusivity was used to solve for the oxygen transport in the fluid phase. In this study, modelling of the steady-state oxygen profile was based on the assumption that the fluid phase oxygen concentration at the free surface is equal to the equilibrium concentration of saturated oxygen at the defined system temperature (37°C).

C. Assessments of cell phenotypes

Values of cell density in medium volumes were measured at the start and end of three-day culture periods using packed cell volume centrifuge tubes as described previously [3]. Densities were converted to increases in cell biomass per unit volume. Photomicrography was performed using a digital camera attached to an upright stereomicroscope Staining of EL-4 cells with the vital mitochondrial stain rhodamine-123 (Molecular Probes, Eugene, OR, USA) and fluorescence intensity detection of mitochondrial activity in live cells was performed using digital fluorescence microscopy, as described previously for hemopoietic stem cells [5]. Mean triplicate values were expressed in arbitrary units on a scale of 0-256, following background correction. Quantitation was performed using Scion Image Software (Scion Image Corp., Frederick, MD, USA).

D. Assessment of medium composition

Lactate/glucose ratios were calculated from individual levels measured using spectrophotometric assays, as described previously [3]. Levels of dissolved oxygen in RAD culture media were measured directly, to confirm theoretical evaluations of oxygen distribution. Measurements were made using a portable polarimetric dissolved oxygen (DO) meter (YSI, Yellow Springs OH, USA, model no. DO200). The probe tip was mounted from beneath the bottom surface of a test well via central access port, with the edges of the sensor sealed to prevent medium leakage during measurement. Continuous readings of DO (mg/L) were obtained during a simulated RAD culture, and compared to stabilized values in static medium, both performed at two independent time points in three replicate experimental groups. All measurements were taken within the culture incubator under similar conditions used for cell propagation.

E. Statistical evaluation

Mean values of cell biomass increase, log-transformed ratios of lactate-to-glucose, fluorescence intensities and dissolved oxygen levels were compared using an unpaired t-test. Differences between means with a p-value of 0.05 or less were considered biologically significant.

III. RESULTS

A. Oxygen diffusion in the RAD bioreactor

Computational prediction of oxygen concentration in RAD cultures at the chosen rotational speed revealed a near homogenous redistribution of dissolved oxygen throughout the culture medium, compared to static medium (Fig. 2). The centre of the recirculation zone was slightly lower in dissolved oxygen, due to a relatively less efficient diffusion between neighbouring fluid layers emanating from the slow anticlockwise flow, radially outward from the central more well-oxygenated area. In the RAD vessel, dissolved oxygen concentrations at two selected radial positions of r/R = 0.2 were approximately 5% (h/R = 0.27) and 39% (h/R = 0) higher than under static conditions, indicating an efficient transfer of dissolved oxygen from the air-fluid interface to the bottom surface of the well, close to where colonies typically aggregated. Figure 2(b) shows the positive impact made by an increase in the stirring speed in terms of transporting oxygen. The streamlines indicate enhanced transport from the air-medium boundary and central regions to the other sections, following the anti-clockwise pattern of the recirculation. In agreement, absolute levels of dissolved oxygen at a similar position near the RAD vessel base (3.45 mg/L, SEM = 0.03, n = 15) were significantly higher than the same location in static media (2.26 mg/L, n = 15, SEM = 0.02, $p < 0.001$). EL-4 lymphoma aggregates grown in the RAD bioreactor formed macroscopically visible plaques, similar to those described in

longer-term cultures [3], though with a more heterogeneous appearance (Fig. 3). These larger colonies (3-5 μm) re-aggregated centrally along the bottom surface of RAD wells, despite the net movement of fluid away from this region. Surrounding these central plaques were smaller, evenly distributed plaques (approx 0.2-0.4 mm) that visibly decreased in spatial density with increased distance away from the central colony.

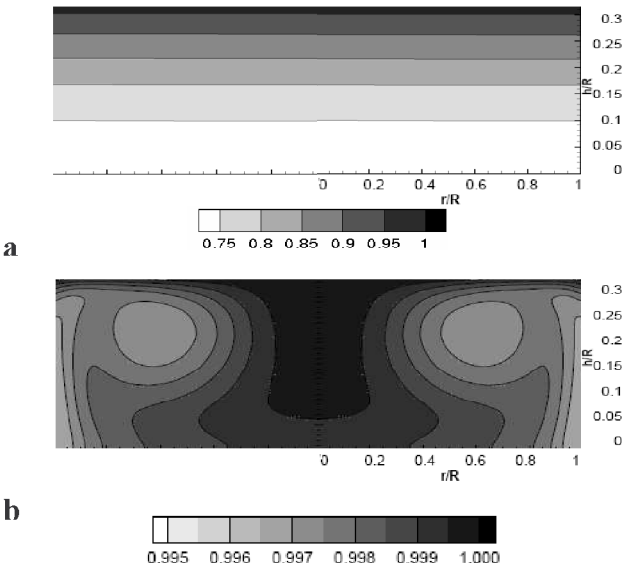

Fig. 2. Predicted contour plots of normalized oxygen concentration in (a) static culture medium, and (b) culture medium with RAD entrainment (r = disc radius, R = well radius, h = height of medium).

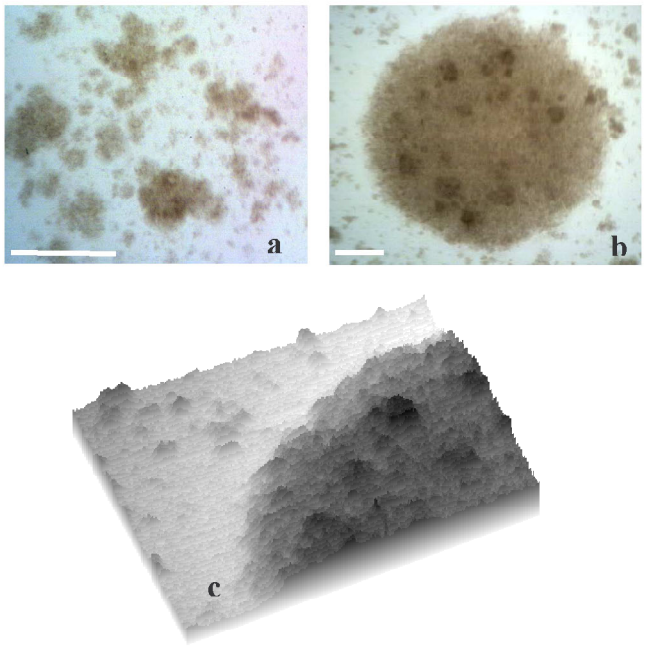

Fig. 3. Light micrographs of EL-4 mouse lymphoma colonies formed in (a) static cultures and (b) in the RAD bioreactor; (c) 3D rendering of a large RAD tumor colony. (Scale bar = 1 mm).

B. Physiological responses to bioreactor culture

Despite the morphological differences in colony distribution and similar proliferation rates, RAD cultures contained significantly lower molar ratios of lactate relative to glucose (1.39 ± 0.02, n = 3) in comparison to static cultures (1.84 ± 0.05, n = 3, $p < 0.05$). This metabolic alteration was associated with reductions in mitochondrial activity, indicated by significantly lower rhodamine-123 intensity in samples of live EL-4 colonies following bioreactor culture (68 ± 4 AU, n = 3) compared to static culture (157 ± 8 AU, n = 3, $p < 0.05$).

IV. DISCUSSION

The rotating aerial disk (RAD) bioreactor is an effective alternative stirred culture method for tumor cell aggregates. In regard to the formation of large tissue aggregates, for possible in vivo applications, the RAD device facilitated the three dimensional colonies formation without scaffolds. This phenomenon of agglomeration been reported for embryonic stem cell colonies cultured at high-densities [6], especially those in close physical contact [7]. While not desirable for the purposes of continuous passaging of that cell type, the assembly of dense colonies simulates native tissue morphology, creating a more physiological culture. Such an approach has been adopted previously in a centrifugal bioreactor to produce solid cancer tissue [8], as well as for controlled high-density seeding of porous scaffolds with cultured stem cells [9]. Indeed, centrifugation to create large, high-density tissue constructs without a bioreactor growth phase is now possible [10].

The limitations of oxygen diffusion through large medium volumes and into dense tissues or porous scaffold/tissue constructs inside bioreactors has been addressed by a range of gas delivery modes, including direct dispersion, sparging, bubbling, perfusion of pre-oxygenated medium, delivery of oxygen binding agents, and direct vessel agitation [11]. Many of these approaches result in excessive shear stress in the vicinity of cells. The RAD bioreactor uses an alternative approach to improve the theoretical oxygen mass transfer rate [12] by increasing the driving force, the medium surface area, and the distance of depth-wise diffusion by maintaining that used in static cultures, which support cell survival by passive diffusion. Also, the laminar flow vortex pattern results in a uniform recirculation of oxygen from the air-medium interface down to the vessel base, as confirmed by the CFD and DO results. This approach has advantages of over other stirred bioreactors in terms of its generally lower levels of turbulence and stress/strain, and robustness of steady-state flow. Indirect mixing by vessel agitation, such as orbitally-shaken culture plates, may be a seen as less complex alternative to the RAD in operation, however such a design has several operational difficulties in terms of scale-up and power requirements (i.e. all wells stirred simultaneously). While volumetric mass transfer rates in such devices has been described as comparable to stirred bioreactors [13], high aspect ratio volumes are required, the fluid flow is generally chaotic and can be subject to flocculation, conditions to which homogenously suspended bacteria or yeasts are more resistant than shear sensitive mammalian cells [14]. The RAD design is

a relatively simple, low-power, controllable device, suited to fixed tissues or scaffold/tissue constructs, rather than homogeneous suspensions, particularly using the shallow medium arrangement. Further investigations of oxygen transfer into cell colonies and scaffold/tissue constructs as a result of the improved oxygen delivery remains to be performed.

Biomechanical stimulation and re-modelling of colony growth is relevant to emerging research in cancer biology. Engineering tissue constructs are currently providing novel three-dimensional tumor models for in vitro applications [15]. Further investigation of the corresponding rheological properties of cancer tissue (eg. elasticity or stiffness) cultured under variable flow conditions may provide additional insights of potential diagnostic value [16]. Such assessments can be readily achieved experimentally using imaging in parallel with variable flows in the RAD vessel.

Regulation of tumor growth by metabolic processes is also undergoing a renewal of interest as far hypoxic response and the Warburg Effect [17]. Culture of tumor colonies in the RAD resulted in a reversal of this effect, as a direct result of improved oxygen diffusion, in contrast to the hypoxic response described previously [3]. The decreased mitochondrial activity in EL-4 cells following RAD culture further supports a shift to a more quiescent metabolic state, in agreement with previous evidence that mitochondria in many cultured cancer cell lines are hyper-activated [18]. Despite being regulated mainly by anaerobic glycolysis, the EL-4 cell line has been shown to retain the ability to modulate mitochondrial respiration in response to variable substrate supply [19]. Similar metabolic plasticity has been observed in other cancer cell lines in response to modifications in metabolic substrate supply [20, 21]. Martin and colleagues showed dramatic alterations in intracellular ATP synthesis and glycolytic metabolism, despite little change in cell proliferation [21], in some agreement with our observations.

CONCLUSION

The RAD bioreactor represents a suitable alternative or add-on to static tissue culture vessels, as well as for larger scale applications because of its scalable geometry. The same method could also be extended any anchorage-dependent cells type adapted as suspended spheroids [22], or bound to microcarrier particles [23]. Further investigations of the technique in drug delivery in models of solid tumors of different scales would also be a relevant application in cancer research.

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