Bioreactor Development for Stem Cell Expansion and Controlled Differentiation

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Summary of Recent Advances

Widespread use of embryonic and adult stem cells for therapeutic applications will require reproducible production of large numbers of well-characterized cells under well-controlled conditions in bioreactors. During the last two years, substantial progress has been made towards this goal. Human mesenchymal stem cells expanded in perfused scaffolds retained multi-lineage potential. Mouse neural stem cells were expanded as aggregates in serum-free medium for 44 days in stirred bioreactors. Mouse embryonic stem cells expanded as aggregates and on microcarriers in stirred vessels retained expression of stem cell markers and could form embryoid bodies. Embryoid body formation from dissociated mouse embryonic stem cells, followed by embryoid body expansion and directed differentiation, was scaled-up to gas-sparged, 2-liter instrumented bioreactors with pH and oxygen control.

Introduction

Embryonic and adult stem cells are defined by their ability to either self-renew or differentiate into multiple cell lineages. These properties make stem cells attractive as a cell source for cell therapies, tissue engineering, and model systems for drug screening [1]. Successful implementation of stem-cell-based technologies will require the ability to generate, large numbers of cells with well-defined characteristics. Depending on the application, culture systems must be designed to (1) produce expanded stem cells with uniform properties and/or (2) promote controlled, reproducible differentiation into selected mature cell types of high purity.

The key to successful large-scale, long-term cultures is the development of well-defined, controlled conditions for specific culture outcomes. Stirred and perfused bioreactors have a significant advantage over static culture vessels due to the more homogeneous environment and the ability to monitor and control critical culture parameters. However, many challenges remain. For example, the need to provide cells with an adequate oxygen supply must be balanced against the detrimental effects of hydrodynamic shear stress developed in stirred and gas-sparged reactors. Sensitivity to the build-up of metabolic byproducts and associated

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changes in pH must also be considered. Also, while parameters such as oxygen partial pressure (pO$_2$), pH, and shear stress affect all types of cells, the optimal parameter values will differ for different cell types and objectives (e.g., expansion vs. differentiation). In this review we focus on recent developments (January 2005 to March 2007) in the use of bioreactors for stem cell cultures. We also discuss studies in static culture systems that examined the effects of key parameters and therefore contribute to the development of better defined conditions for stem cell culture in bioreactors.

Stem Cell Expansion and Differentiation in Bioreactors

During the past two years, many studies have evaluated the expansion and/or differentiation of embryonic and adult stem cells using a variety of bioreactors (Table 1). Hematopoietic stem and progenitor cells (HSPCs) were among the first stem cells to be cultured in bioreactors. Two recent studies have confirmed that HSPC expansion is more extensive in stirred or suspended culture [2,3]. In contrast to HSPCs, most bioreactor studies with mesenchymal stem cells (MSCs) have employed perfusion of cells embedded within a 3-dimensional (3-D) scaffold. Perfusion produced a more uniform distribution of human or sheep MSCs within the scaffolds [4,5,6•], and MSC expansion was accomplished without loss of multi-lineage differentiation potential [5,7,8]. Also, human bone marrow stroma stem cells expanded and differentiated in perfused 3-D scaffolds were more effective at forming bone after implantation in mice [9].

Several types of tissue-specific stem cells have been successfully cultured as aggregates in stirred reactors using defined serum-free media [10,11••,12,13••,14]. Mouse neural stem cells were expanded in spinner flasks for up to 44 days with subculturing every four days [10]. The growth rate was similar throughout the culture and the expanded cells retained multi-lineage neural differentiation potential *in vitro* and the ability to engraft *in vivo*. Mouse neural stem cell culture was scaled up to 500 mL in an instrumented and controlled bioreactor [11••]. Mouse mammary epithelial stem cells were expanded as aggregates (or mammospheres) in spinner flasks [12]. Mouse breast cancer stem cells expanded as aggregates in an instrumented and controlled stirred bioreactor retained the potential for clonal growth, including secondary colony formation [13••]. Culturing pig neonatal pancreatic tissue in spinner flasks led to the production of islet-like aggregates [14]. Although the number of cells decreased during the 9-day culture, the fraction of endocrine cells and the number of insulin-positive cells increased.

Many research groups have employed bioreactors for embryonic stem cell (ESC) expansion and/or differentiation. Expansion of undifferentiated mouse ESCs (mESCs) is conducive to bioreactor culture because feeder layers and conditioned medium are not required if leukemia inhibitory factor (LIF) is provided. Greater expansion in stirred vs. static systems was demonstrated for mESCs cultured as aggregates [15,16••] and on microcarriers [16••,17•]. The expanded mESCs retained expression of ESC surface markers [15,16••,17•] and the ability to form embryoid bodies [15,16••]. zur Nieden et al. showed that mESCs could be maintained as aggregates in suspension culture for at least 28 days (seven passages) [18•]. mESCs within the aggregates were similar to input mESCs with regard to ESC surface marker expression, embryoid body formation potential, *in vitro* differentiation into cells types from all three germ layers, and *in vivo* teratoma formation. Cell-cell interactions between mESCs in aggregates and on microcarriers appear to be mediated in large part by the cell adhesion molecule E-cadherin [16••].

Embryoid body (EB) formation from ESCs and the directed differentiation of EBs are typically carried out in static cultures. However, several groups have recently shown that mouse EBs can be formed directly from enzymatically dissociated mESCs in stirred reactors [19••] or a rotary cell culturing system [20]. Mouse EBs can also be formed in stirred vessels by removing

LIF from mESC aggregates produced in the same vessels [16••] or by encapsulating mESC aggregates in agarose beads [21••]. After formation, the EBs grew in size and retained the potential for cardiomyocyte differentiation [19••,21••]. Encapsulating EBs has the advantage of preventing EB aggregation. Less extensive mouse EB aggregation can also be obtained by forming the EBs on tantalum scaffolds suspended in a spinner flask [22].

Expansion of undifferentiated human ESCs (hESCs) is more difficult than for mESCs, and has not yet been reported in stirred cultures. LIF does not support the expansion of undifferentiated hESCs, so feeder layers or conditioned medium are typically required. Two recent reports of feeder-free hESC expansion in defined, serum-free media on surfaces coated with laminin [23] or fibronectin [24] improve the prospects for hESC expansion in stirred reactors. In contrast to undifferentiated hESCs, preformed human EBs have been expanded in stirred vessels. Human EBs cultured in stirred vessels exhibited greater cell expansion, more uniform morphology and size distribution, and a similar potential to differentiate into hematopoietic cells, as compared to EBs cultured in T-flasks [25].

Shear

Cells in stirred bioreactors are exposed to hydrodynamic shear stress. In the absence of gas bubbles, damage to single cells in suspension or to cells in aggregates or on microcarriers occurs when the turbulent Kolmogorov eddy size has the same order of magnitude as the diameter of a single cell, aggregate, or microcarrier [26]. Since the Kolmogorov eddy size decreases with increasing agitation intensity, cells on microcarriers or in aggregates are affected at much lower agitation rates than single cells. The hydrodynamic shear stress increases with increasing impeller diameter and rpm and also depends on the impeller geometry and location. The shear stress is also increased by the presence of probes and other vessel internals that disrupt radial flow patterns [11••]. Gilbertson et al. showed that stirred cultures of neural precursor cell aggregates can be scaled-up based on the calculated maximum shear stress [11••]. There is an optimal shear stress range for aggregate and embryoid body cultures, with extensive clumping at very low shear stress and cell death at high shear stress [12,13••,15, 16••,19••]. Also, stagnation zones that result from suboptimal impeller placement were shown to promote embryoid body aggregation [19••]. Different stem cell types have different optimal shear stress values. For example, the optimal shear stress for mammary epithelial stem cell aggregate culture was 0.21 Pa [12]. In contrast, mESC aggregate cultures had an optimal shear stress of 0.61 Pa, and exhibited extensive clumping at a shear stress of 0.45 Pa [15]. Encapsulation of mESC aggregates in agarose allowed for embryoid body formation and expansion at high agitation rates [21••].

Most stem cell cultures in stirred reactors have been carried out using maximal cell densities and culture volumes that allow for adequate oxygen transfer from the headspace. However, sparging with gas bubbles will be required to achieve adequate oxygen delivery in large-volume, high-cell-density reactors. Sparging causes cell damage because cells attach to the gas bubbles and are subject to high shear stress during bubble rupture [26]. Also, sparging of high-protein-content media produces extensive foam that decreases reactor capacity. Surface-active anti-foams can also minimize cell damage by decreasing cell-bubble attachment, but must be evaluated for stem-cell toxicity. In a significant advance, Schroeder et al. showed that mESC viability was not affected by low concentrations of Antifoam C, and used 0.0125% Antifoam C to successfully scale-up mESC embryoid body formation, expansion and differentiation to a 2-L stirred, sparged, and instrumented reactor with pH and pO_2 control [19••].

Oxygen Tension

Studies in static culture systems have generally shown that low pO_2 decreases the rate of stem cell differentiation and may also enhance stem cell proliferation. For example, human [27] and

mouse [28] ESCs were reported to expand more rapidly under 2–5% vs. 20% O_2, and hESC differentiation was decreased under 3–5% vs. 20% O_2 [29]. After a short lag period, human MSCs grew more rapidly under 2% vs. 20% O_2 and exhibited greater retention of MSC markers and formation of CFU-F when cultured at 2% O_2 [30]. Bone-marrow-isolated adult multi-lineage-inducible (MIAMI) cells also exhibited greater expansion, retention of ESC markers, and decreased osteogenic differentiation under 3% vs. 20% O_2 [31]. Low pO_2 protected hESCs by decreasing chromosomal damage, but also sensitized the hESCs to oxidative damage after a shift back to 20% O_2 [27]. MIAMI cell expansion was less extensive under 1% vs. 3–10% O_2, although it was still greater than under 20% O_2 [31]. This was likely due to oxygen depletion under 1% O_2 and emphasizes the point that pO_2 experienced by cells in static cultures is less than that in the gas phase. It should also be noted that pO_2 within cell aggregates or scaffolds decreases rapidly with the distance from the bulk liquid interface [6•,13••].

Perfusion

Continuous perfusion with fresh or recycled medium increases oxygen transport to cells in culture dishes or within scaffolds. Several investigators reported that perfusion increases MSC density within the interior of 3-D scaffolds [4,5,6•]. Perfusion or frequent feeding also enhances culture performance by replacing depleted nutrients and/or removing inhibitory metabolic byproducts. For example, Cormier et al. attributed the decline in viability of mESC aggregates after 6 days in batch culture to the associated build-up of ammonia (to 2.5 mM) and decrease in pH (from 7.6 to 6.6) [15]. Perfusion, frequent feeding, and/or the use of gas-permeable culture surfaces increased the expansion of MSCs [4,5,6•], ESCs [32,33] and mammary epithelial stem cells [12], while retaining stem cell potential [5,9,32,33]. Under conditions for which oxygen was not limiting, Zhao et al. reported greater human MSC expansion at a lower perfusion rate [7]. Less extensive growth at the higher perfusion rate was attributed to adverse effects of the greater hydrodynamic shear stress. However, since the shear stress was very low, it is also possible that growth inhibition was due to greater removal of growth-promoting factors produced by the MSCs (see [34] for a discussion of interactions between protein secretion and interstitial fluid flow). In other cases, stem cell expansion can be increased by removing inhibitory cytokines produced by their more differentiated progeny, as has been demonstrated for hematopoietic stem cells [35,36].

Conclusions

Studies from the last two years have demonstrated the potential to culture human MSCs and HSPCs, as well as several types of mouse stem cells, for prolonged periods in several types of bioreactors at volumes up to two liters. Expansion of other types of human stem cells in bioreactors under defined conditions remains a challenge. However, the prospects for expansion of undifferentiated hESCs in bioreactors have been enhanced by (1) reports of prolonged mESC expansion in bioreactors and (2) the identification of culture media/cytokine combinations and extracellular matrix components that allow for feeder-free expansion of undifferentiated hESCs in static culture.

Future challenges include the development of optimal culture conditions for various stem cell types and scale-up to large-volume production systems. The benefits of perfusion and frequent feeding have been confirmed by many recent studies. However, optimal values for many culture parameters remain to be identified. For example, it was recently shown that rat MSC differentiation and protection from apoptosis are enhanced at 32°C vs. 37°C [37]. The effects of pO_2, pH, temperature, and nutrient concentrations have primarily been investigated in static cultures. However, optimal values for these parameters must be determined in bioreactors under well-defined and controlled conditions. It should also be noted that optimal parameter values will differ for different types of stem cells, as well as for stem cell expansion vs.

differentiation to various cell types. Shear effects have been examined for several types of stem cell aggregates, but more detailed research should be carried out for aggregates, as well as for microcarrier culture. Supplying sufficient oxygen to large-scale reactors represents a major challenge and will likely require gas sparging, so it was encouraging to see that a mESC-compatible antifoam allowed for pO_2 control via gas sparging in embryoid body cultures. Similar studies remain to be conducted for other types of stem cells.

Acknowledgements

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Table 1

Stem cell culture in bioreactors.

Reference	Bioreactor	Species	Stem Cell Type	Purpose	Notes
[2]	Rotating Wall Vessel	Human	HSPC	Expansion	pH & osm. offline*
[3]	Spinner Flask	Human	HSPC	Expansion	
[4]	Perfusion	Sheep	MSC	Expansion	glu. offline
[5]	Perfusion	Human	MSC	Expansion	glu. & lac. offline
[6•]	Perfusion	Human	MSC	Expansion	pH, pO$_2$, glu. & lac. offline
[7]	Perfusion	Human	MSC	Expansion	
[8]	Rotary Cell Culture System	Human	MSC	Expansion	
[9]	Perfusion	Human	BM Stromal	Expansion	
[10]	Spinner Flask	Mouse	Neural	Expansion	
[11••]	Instrumented Stirred Vessel	Mouse	Neural	Expansion	T, pH, & pO$_2$ control. pH, osm. offline
[12]	Stirred Vessel	Mouse	Mammary Epithelial	Expansion	
[13••]	Instrumented Stirred Vessel	Mouse	Breast Cancer	Expansion	T, pH, & pO$_2$ control; glu., lac., gln., & amm. offline
[14]	Spinner Flask	Pig	Neonatal Pancreatic	Expansion	
[15]	Spinner Flask	Mouse	ESC	Expansion	pH, glu., lac., gln., & amm. offline
[16••]	Spinner Flask	Mouse	ESC	Expansion	
[17•]	Spinner Flask	Mouse	ESC	Expansion	glu. & LDH offline
[18•]	Spinner Flask	Mouse	ESC	Expansion	Same as [15]
[19••]	Instrumented Stirred Vessel	Mouse	ESC	Differentiation	T, pH, and pO$_2$, control; gas sparging
[20]	Rotary Cell Culture System	Mouse	ESC	Differentiation	
[21••]	Instrumented Spinner Flask	Mouse	ESC	Differentiation	Continuous perfusion; pH & pO$_2$ control
[22]	Spinner Flask	Mouse	ESC	Differentiation	
[25]	Spinner Flask	Human	ESC	Differentiation	

Abbreviations: amm. = ammonia, BM = bone marrow, ESC = embryonic stem cell, gln. = glutamine, glu. = glucose, HSPC = hematopoietic stem and progenitor cell, lac. = lactate, LDH = lactate dehydrogenase, MSC = mesenchymal stem cell, osm. = osmolality, pO2 = oxygen partial pressure, T = temperature