3D culture of mouse gastric stem cells using porous microcarriers

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1. ABSTRACT

The lining epithelium of the stomach includes multipotent stem cells which undergo proliferation and migration-associated differentiation. These cells give rise to multiple cell lineages that produce mucus, acid, pepsinogen and various hormones/peptides. A 3D culture for stem cells would facilitate identification of the factors that control proliferation and/or differentiation programs. Here, we report on the use of disk-like ImmobaSil HD silicone-rubber matrix based microcarriers that are permeable to oxygen and reduce the creation of toxic environment within the center of the microcarrierd for culturing the mouse gastric stem (mGS) cells. We define several parameters that affect the initial cell attachment such as size of cell inoculum, serum concentration, mode and speed of agitation. We show that although such a microcarrier allows for attachment and growth of gastric stem cells, it does not lend itself and does not support the functional differentiation of such cells.

2. INTRODUCTION

The gastric epithelium is organized to form numerous tubular glands. They contribute to stomach function by producing the gastric juice and several hormones. To maintain the integrity and continuous function of these glands, their lining cells renew by division of a small population of multipotent stem cells located in a strategic mid-position of each gland. These adult stem cells are small in number, thus making their isolation and expansion difficult but important to facilitate the understanding of their roles in gastric epithelial homeostasis, development of gastric cancer, and the emerging field of gastric tissue engineering (1). Gastric cancer is the third leading cause of death among the various types of cancer (2). The use of gastric stem cells in tissue engineering will benefit many gastric cancer patients undergoing gastrectomy as part of their postoperative procedures (3,4).

Conventional methods of culturing anchorage-dependent animal cells require the use of flat tissue-culture T-flasks and the frequent need of passaging and expanding the culture upon reaching certain confluency. Due to the limited surface area, these methods are time consuming and highly susceptible to contamination. Producing scaled-up animal cell cultures

using microcarriers was first described in 1967 by van Wezel (5). This culture system allows cells to attach onto a small solid particle that floats in the culture medium with gentle agitation. Microcarriers have been previously used for scaling up the production of viral vaccines and recombinant therapeutics (6). In addition to a scaled-up cell culture as a result of the larger surface area, porous microcarriers provide a three-dimensional (3D) microenvironment that better mimics cells *in vivo* than the two-dimensional (2D) microenvironment observed in a monolayer culture (7). Since 1967, intensive research has demonstrated the successful culture of cells, such as CHO cells (8), mesenchymal stem cells (9,10), human islet cells (11), fetal stem cells (12), and chondroprogenitor cells (9), using microcarriers with various morphologies, biomaterial compositions, and culture methodologies (7).

Scaling up the production of cells is also important for stem cell cultures. The aim of differentiating stem cells into specialized cells for clinical applications requires a large quantity of cells (13); for example, up to 6 billion mesenchymal stem cells may be used in clinically treating osteogenesis imperfecta (14). In addition to their expansion, the differentiation of stem cells maintained in a microcarrier culture system was reported by several groups using embryonic stem cells (13) and mesenchymal stem cells (14,15). It has been shown that the biochemical and mechanical properties of microcarriers regulate decisions of stem cell fate (16).

Both solid-filled and porous microcarriers are used to culture cells (7). Solid-filled microcarriers are suitable for scaling up anchorage-dependent cell cultures but have smaller surface areas than porous microcarriers, exhibit higher shear stress, and can form aggregates (17). Porous microcarriers, on the other hand, have several advantages including their 3D topography with a large surface area allowing the use of fewer microcarriers and therefore, protecting cells from shear stress and damage. This also allows the use of higher stirring speeds (17,18).

Despite the many advantages of using porous microcarriers, a diffusion issue might arise (17,18). Cultured cells consume nutrients, such as glucose, and produce metabolites, such as lactate. At either very high or low concentration levels, nutrients and metabolites can inhibit cell growth and consequently should be monitored throughout the culture period (19). However, as the cellular density in the pores of microcarriers increases, gas, nutrients, and metabolite exchange becomes more difficult to maintain between the surface of the microcarrier and its center. This can lead to a toxic environment near the center of the microcarrier, thus decreasing cell numbers (8,17,18,20). To address such issues, microcarrier morphology and biomaterial composition are essential factors directing the fate of the cultured cells (7). Increasing the efficiency and optimizing the cell growth in microcarrier culture system can be achieved

by determining the factors affecting cell attachment onto the microcarrier during the initial few hours of the culture. This should be determined for each microcarrier type and cultured cell type. The factors affecting cell attachment include cell inoculum concentration, agitation speed/mode, and media composition.

In this study, mouse gastric stem (mGS) cells were cultured on disk-like ImmobaSil HD microcarriers. These immortalized epithelial cells were established from a genetically engineered mouse model and found to lack any differentiation markers of gastric epithelium, but express stem cell markers: Notch 3, Dcamkl1, and Oct4 (21,22). Thus they are a good representative of the gastric epithelial stem cells. The ImmobaSil HD microcarriers are silicone-rubber-matrix-based, negatively charged, and hydrophobic with high permeability to oxygen (23). This allows the diffusion of oxygen to the center of the microcarrier. As the literature shows, initial cell attachment is a key factor in the success of a microcarrier culture (24). Here, we determined the parameters affecting cell attachment during the first six hours of cell culture, including cell inoculum, serum concentration, agitation speed, and agitation mode. We also tested the ability of mGS cells to differentiate when cultured on ImmobaSil HD microcarriers by using scanning electron microscopy and fluorescence microscopy with two lectins: *Ulex europaeus* agglutinin 1 (UEA) as a marker for differentiating mucus-secreting pit cells and *Griffonia simplicifolia* II (GSII) which detects mucus-secreting neck cells (21).

3. MATERIALS AND METHODS

3.1. Cell culture

A frozen sample of mGS cells at passage 26 was used in this study. This cell line was previously established and characterized in our laboratory (21). The culture medium, HyClone RPMI 1640 (ThermoScientific, Pittsburgh, PA, USA) was prepared by the addition of 10% (v/v) fetal bovine serum (FBS; ThermoScientific), and 1% (v/v) penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 100 IU and 100 µg/mL, respectively. Prior to experimentation, cells were cultured at 37°C with 5% CO_2 until nearly confluent (Figure 1A). Cells were then harvested by first washing twice with phosphate buffer saline (PBS) followed by the addition of 0.2.5% trypsin to enzymatically detach cells. The mGS cells were counted with a hemocytometer using the trypan blue (Sigma-Aldrich) exclusion method.

3.2. Preparation of microcarriers

ImmobaSil HD microcarriers were obtained from Ashby Scientific (Leicestershire, UK). They have a diameter of 0.3. to 0.8 mm and pores of size 50X150 µm, making up more than 40% of the microcarrier. We added 1 gm (dry weight) of the microcarriers to 100 ml shaking flasks. Deionized water obtained from a Milli-Q water
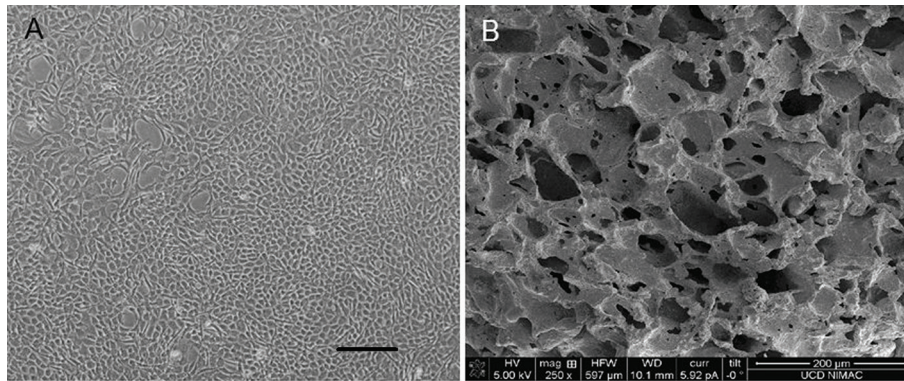
Figure 1. Microscopic appearance of mGS cells and ImmobaSil HD microcarriers used in this study. A. Phase contrast micrograph of mGS cells before trypsinization for seeding on microcarriers. B. Scanning electron micrograph of ImmobaSil HD microcarrier. Magnification bar in A =100 µm.

purification system was added to the flasks until the microcarriers were completely submerged. Flasks with microcarriers were autoclaved at 120°C for 30 minutes for sterilization. For the cell attachment experiment, water used in autoclaving the microcarriers was replaced with cells inoculated at densities 10×10^5, 8×10^5, 4×10^5, and 2×10^5 cells/ml. The initial volume of the culture was 20 ml, but after the initial six hours of culture, the volume was raised to 50 ml. FBS concentration, agitation speeds, and modes were set depending on the experimental parameters. FBS concentrations ranged from 0, 5, 10 and 15%. Agitation speeds were set at 70,110, and 140 rpms, whereas agitation modes were set at 15 minutes stirring/15 minutes no stirring, 20 minutes stirring/40 minutes no stirring, 10 minutes stirring/50 minutes no stirring, and finally continuous stirring. Microcarrier cultures were maintained at 37°C in 5% CO_2. 50% of the media volume was renewed with fresh media every other day. This was done after glucose levels dropped to 50% of original concentrations to avoid increased media toxicity.

3.3. Metabolic analysis

For the glucose and lactate concentration measurements, 50 µl samples of the culture media were collected into Eppendorf tubes. Samples were taken before and after culture media change. Samples were centrifuged at 400g to eliminate any debris present in the media. 10 µl of the supernatant was pipetted onto glucose measurement strips using a glucose meter (Bayer, Leverkusen, Germany). Results were recorded in mmol/L. For the lactate concentration measurement, 10 µl of the centrifuged supernatant was pipetted onto lactate measurement strips using Accutrend lactate meter (Roche, Mannheim, Germany). Results were recorded in mmol/L. All values were given as the mean of three samples. Standard deviation was calculated to present error bars.

3.4. Scanning electron microscopy

ImmobaSil HD microcarriers were first washed in PBS and fixed in 3.7.% paraformaldehyde in 1X

PBS (Sigma-Aldrich, St. Louis, MO, USA). Samples were then dehydrated in ascending grades of ethanol solutions (from 20 to 100%) and then treated with hexamethyldisilazane (Sigma-Aldrich, St. Louis, MO, USA) as a drying agent. This was followed by coating with gold/palladium using a sputter coater (Ted Pella, Redding, CA, USA). A scanning electron microscope (SEM; Philips, Eindhoven, Netherlands) was used to observe the samples.

3.5. Fluorescence labeling

ImmobaSil HD microcarriers with attached cells were washed once with 1X PBS then fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes. Following three PBS washes, the cells attached to microcarriers were incubated for 5 min with 300 nM 4',6-diamidino-2-phenylindole dilactate (DAPI; Invitrogen) prepared in water. An Olympus inverted microscope connected to DP70 CCD camera (Tokyo, Japan) was used to observe the samples. A fixed intensity time (500 ms) was set to observe both samples and negative controls.

3.6. Differentiation experiment

To test whether the 3D microcarrier culture system would support cellular differentiation, the mGS cells were cultured on microcarriers for 21 days. In some experiments, the RPMI media of microcarrier cultures was changed on day 14 with human gastric stem cell differentiation media with fetal bovine serum (Celprogen Inc., Torrance, CA, USA). The cultured cells were maintained at 37°C in 5% CO_2, and media was replaced with fresh media every other day. Samples of microcarriers with attached cells were processed on day 21 for SEM examination as mentioned above and lectin-binding analysis.

Lectin staining was utilized to detect any signs of mucous cell differentiation in cultured mGS cells. The mGS cells cultured on ImmobaSil HD microcarriers were first fixed for 15 minutes with 3.7.% paraformaldehyde,
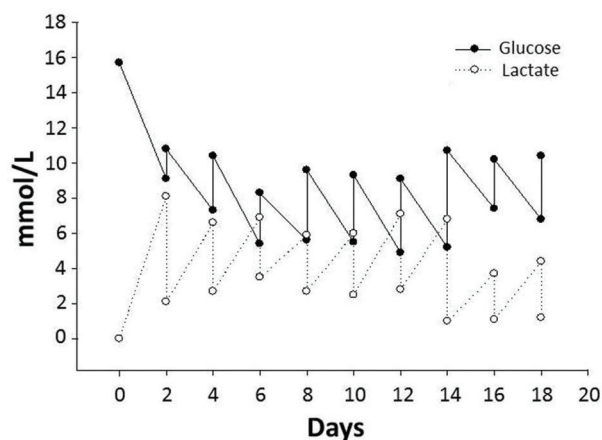
Figure 2. Metabolic activity of mGS cells cultured on microcarriers. Levels of glucose and lactate were measured before and after media changes over 18 days of culture. Note that $2X10^5$ cells/ml media and 1 gm of microcarriers were used.
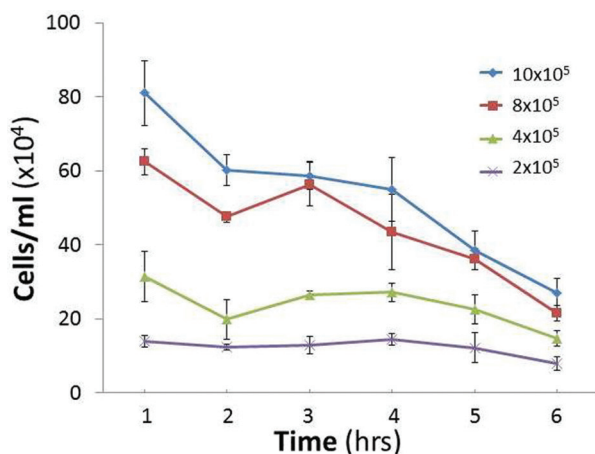

Figure 3. Effect of cell inoculum on cell attachment. The mGS cells at $10X10^5$, $8X10^5$, $4X10^5$, and $2X10^5$ cells/ml were used with 1 gm of microcarriers. The numbers of unattached cells were counted every hour during the initial six hours of incubation.

washed three times with PBS, and then permeabilized for 10 minutes using 0.5.% triton X-100 in 1X PBS. Cells were then incubated for 45 minutes in 3% bovine serum albumen blocking reagent, followed by 60-minute incubation at room temperature with two fluorophore-conjugated lectins: tetramethylrhodamine (TRITC)-UEA (Sigma) at 1:50 dilution and fluorescein isothiocyanate (FITC)-GSII (Sigma) at 1:50 dilution. Cells were then washed three times with PBS, counterstained with Hoechst at 1:10,000 dilution, and mounted using the Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

4. RESULTS AND DISCUSSION

Since the introduction of microcarriers in 1967 by van Wezel (5), several studies have demonstrated

different methods to increase the efficiency of scaling up cell cultures using microcarriers of various properties. Here, ImmobaSil HD microcarriers were used to culture the mGS cells. These microcarriers are silicone-based and microporous (Figure 1B). The metabolic profile of glucose consumption and lactate production by the cultured cells is presented in (Figure 2). The inverse correlation between the levels of glucose and lactate is apparent. Every two days of culture, before changing media, there is a drop of glucose level which is associated with an increase in the level of lactate indicating viability of the cells. There was no toxicity due to lactate production. Factors affecting cell attachment onto the ImmobaSil HD microcarriers were tested, including initial cell inoculum, serum concentration, agitation speed, and agitation mode.

4.1. Effect of cell inoculum density on mGS cell attachment and growth

To investigate the effect of cell inoculum on cell attachment, cell densities of $2X10^5$, $4X10^5$, $8X10^5$, and $10X10^5$ cells/ml were incubated with 1 gm of microcarriers. The initial volume of the culture was 20 ml and, by the end of the initial six hours, the volume was raised to 50 ml. Previous results showed that starting a microcarrier culture with decreased volume enhanced initial cell attachment, thereby improving the final yield (25). Agitation was kept constant at 110 rpm, just enough to keep the microcarriers in suspension. Results showed that, during the initial six hours of culture, lower seeding densities were associated with higher percentages of unattached cells. The optimum cell density was $8X10^5$ cells/ml where only 26.8.% of the cells remained unattached (Figure 3). This correlates with previous work showing that a minimum seeding density is required for a successful cell culture on the microcarriers (26-28). The use of low cell densities may result in a low cell growth rate (26). To overcome the low growth rate for low cell densities, one can use improved media containing adjusted nutritional concentrations that support cell growth for low cell inoculum densities (26).

4.2. Effect of serum concentration on cell growth

In order to investigate the effect of serum concentration, increasing amounts of serum (0, 5, 10, and 15%) were added to the media and incubated with 1 gm of microcarriers. Cell inoculum was $8X10^5$ cells/ml and agitation speed was set at a constant 110 rpm. After the initial 6 hours of culture, samples with 0% serum showed 13.5.% of unattached cells. This was the highest percentage of unattached cells of all the concentrations. Serum concentrations of 5, 10, and 15% did not show significant differences regarding unattached cell percentage. However, as the serum concentration increased, the viability of cells increased. Using the trypan blue exclusion method, serum concentrations of 10 and 15% showed higher cell viability levels than concentrations of 0 and 5% (Figure 4). This result was not unexpected in the light of the fact that adding serum in media assists in
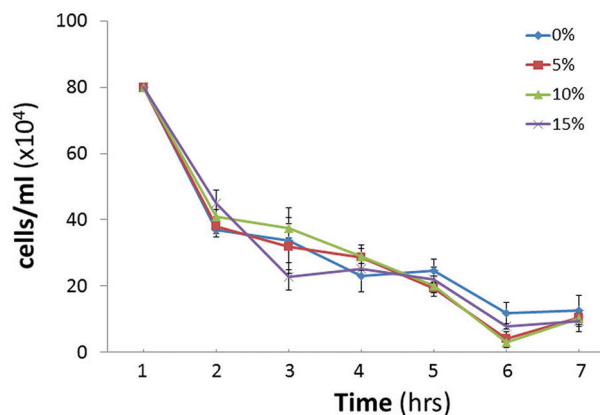
Figure 4. Effect of serum concentration on cell attachment. RPMI culture media containing serum at concentrations of 0, 5, 10, and 15% were inoculated with $2X10^5$ cells/ml and 1 gm microcarriers. The numbers of unattached cells were counted during the initial six hours of incubation.

cell attachment and viability enhancement. This was also demonstrated by work done on human endothelial cells showing that using a 10% serum concentration rather than 5% resulted in a better final cell yield (27). Although the 5, 10, and 15% serum concentrations did not show significant differences in unattached cell percentages during the initial six hours, the long-term effect of the serum concentration change has yet to be examined.

4.3. Effect of agitation speed and mode on cell growth

In order to determine the optimum agitation speed of the microcarrier culture in flasks, different agitation speeds were tested and the results were determined for the initial 6 hours of culture. Speeds of 70, 110, and 140 rpm were used while keeping other parameters, such as the concentrations of microcarriers, inoculum, and serum, constant at 1 gm, $8X10^5$ cells/ml, and 10%, respectively. Results show that the lower the agitation speed, the better the attachment of cells on the microcarriers. When agitated at 70 rpm, only 3.75% of cells remained unattached. This percentage increased when using 110 rpm, which yielded 10.6.% unattached cells, while 140 rpm resulted in the highest percentage of unattached cells at 24.6.%. In addition to cell attachment, vigorous agitation affects the viability of cells. This was seen when cultures agitated at 140 rpm yielded lower cell viability than those at 70 and 110 rpm (Figure 5A). Microcarrier cultures are affected differently by agitation speeds depending on several factors, including cell type and microcarrier composition. Agitation speeds should be individually determined for every culture. However, lower speeds are generally more suitable for cell attachment (9). Lower agitation speeds create less fluid dynamic shear stress in the culture system and decrease the chances of microcarrier-to-microcarrier, and microcarrier-to-flask collisions, resulting in enhanced attachment and viability (9). This correlates with our results of using

70 rpm speed correlating with improved cell attachment and viability. Nevertheless, it was important to keep the microcarrier culture in suspension. Motion was achieved at 110 rpm but not at 70 rpm. Agitation homogenizes the microcarrier culture, allowing cells to attach to all sides of the microcarrier, as well as aiding in oxygen and CO_2 transfer. In addition to determining the effect of agitation speeds, the agitation mode showed that alternating agitating/rest mode of 10/50 minutes resulted in the lowest percentage of unattached cells (Figure 5B).

4.4. Microscopic features of mGS cells on microcarriers

DAPI nuclear staining was used to determine the distribution of cells on the microcarriers. Results showed that the cellular distribution was not restricted to one area of the microcarriers; cells started heterogeneously occupying the microcarriers and formed colonies dispersed throughout the surface. A heterogeneous distribution of cells might arise from several factors, such as agitation, and metabolite concentrations. By day 21, cells occupied most of the microcarrier surface area (Figure 6A). This is consistent with the fact that silicone has a stable chemistry, and is permeable to oxygen, allowing cell viability, and distribution on the microcarriers (17). In addition to revealing the porous structure of the ImmobaSil HD microcarriers (Figure 1B), SEM confirmed the fluorescence microscopic observations and showed that the colonization of mGS cells on the surface of the microcarrier was not restricted to a single area. It also showed the colonization of the cells in the microcarrier's pores. The epithelial nature of the cells and their attachment with minimal intercellular space is demonstrated (Figure 6C). The distribution of mGS cells on microcarriers correlates with previous work showing CHO cells colonizing the surface as well as the pores of ImmobaSil FS microcarriers (18). ImmobaSil FS microcarriers are similar silicone-based microcarriers, highlighting the stability, non-toxicity, and oxygen permeability of the chemical structure of silicone (29).

4.5. ImmobaSil HD microcarriers support mGS cell attachment, proliferation and growth

Following 14 days of culture of mGS cells on microcarriers using RPMI, human gastric stem cell differentiation media obtained from Celprogen was used for 7 days. After this culture period, cells were examined with fluorescence microscopy and SEM and compared with control cells cultured for 21 days using only RPMI media. Nuclear staining showed a reduction in the number of cells attached to the surface of microcarriers when differentiation media was used (Figure 6A,B). This finding suggests a reduction in cell proliferation in the presence of differentiation media. Since, it is known that cell proliferation is inversely related to cell differentiation (30), the cells were further examined for their possible differentiation. SEM examination confirmed the growth of mGS cells on the surface of microcarriers using RPMI and the reduction in the number of cells when
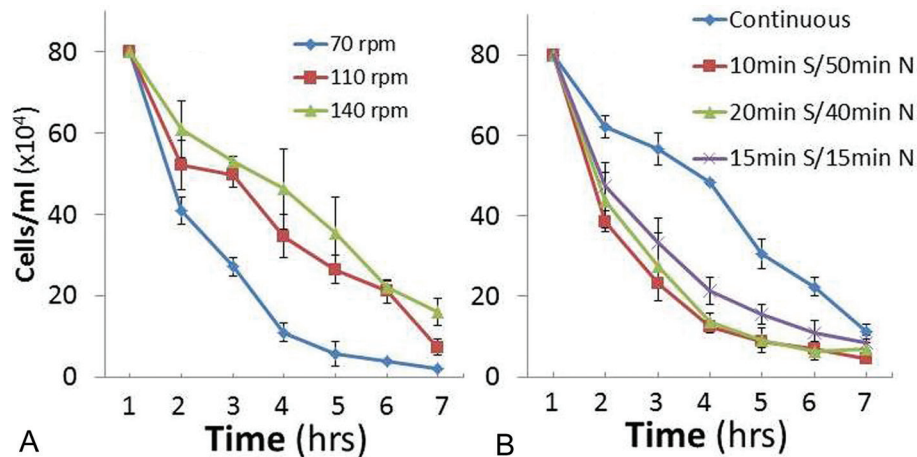
Figure 5. Effects of agitation speeds and modes on cell attachment to microcarriers during the initial six hours of incubation. mGS cells at $2X10^5$ cells/ml media and 1 gm of microcarriers were used. In A, the numbers of unattached cells were counted hourly after using agitation speeds of 70, 110 and 140 rpm. In B, the cells were counted after using different agitation modes.
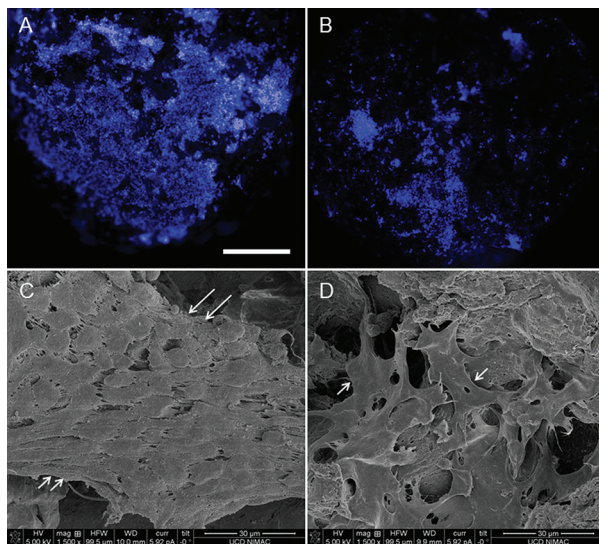

Figure 6. Micrographs of mGS cells attached to microcarriers for 14 days using RPMI media followed by 7 days in RPMI media (A, C) or human gastric stem cell differentiation media (B, D). The cells were visualized after processing for DAPI fluorescence staining (A, B) and scanning electron microscopy (C, D). Compare A and B and note that there are less cells attached to the microcarriers when differentiation media is used. Note the numerous cells in C which extend from upper right to lower left (double arrows). Compare C and D and note the change in the shape and sized of the cells (single arrows) when the differentiation media is used. Also note that in D, the cells at the left arrow extend into the pore on the surface of the microcarrier. Magnification bar for A and B = 100 μm.

differentiation media was added for 7 days. Comparison of electron micrographs also showed an increase not only in intercellular spaces, but also in the cell size (Figure 6C,D). Therefore, the differentiation media induces a reduction in the proliferation of the cells and an increase in their size and intercellular spaces. Flourescence microscopy was also employed to test the possible differentiation into mucous cells using UEA lectin to detect mucus-secreting pit cells and GSII lectin as a marker for differentiation into gland mucous cells. Both cell types are specialized cells derived from mGS cells in the gastric glands. Lectin histochemistry revealed negative staining for both markers. Therefore, while nuclear staining and SEM examination showed successful colonization of mGS cells on the microcarriers with a decrease in their number and increase in their size when differentiation media was used, lectin staining failed to demonstrate that the culture conditions used in this study support the functional differentiation of mGS cells.

5. CONCLUSIONS

The harvesting and culturing of adult stem cells for regenerative medicine has been a challenge due to the high number of cells required. In this paper, mGS cells are used as a model system for growth of gastric stem cells on microcarriers using agitation flasks. It shows the successful expansion of mGS cells on ImmobaSil HD microcarriers. These silicone-based porous, flat-disk-shaped microcarriers provide a large surface area to which cells can adhere. Several important factors affect cell attachment on microcarriers, including cell inoculum, serum concentration, and agitation. Results show that a cell inoculum of $8X10^5$ cells/ml produced better cell attachment on the microcarriers during the first six hours, indicating that a minimum cell concentration is required for an efficient microcarrier culture. Using 10 and 15% serum concentration yielded better cell attachment than lower concentrations. The serum might have encouraged interaction and attachment between cells and microcarriers. Agitation results showed that 110 rpm and intermittent agitation of 10/50 minutes of agitation/rest, respectively, gave better attachment results than higher continuous agitation. Lower speeds that still allow the culture to be in suspension are preferably used, due to the lower shear stress produced in the culture. Although successful growth

of mGS cells on microcarriers was demonstrated, future work will concentrate on modifying this culture system to stimulate functional differentiation of mGS cells.

6. ACKNOWLEDGEMENTS

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Abbreviations: 3D: three dimensional; mGS cells: mouse gastric stem cells; TRITC: tetramethylrhodamine; UEA: *Ulex europaeus* agglutinin 1; FITC: fluorescein isothiocyanate; GSII: *Griffonia simplicifolia* II

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