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Effects of Glutamine, Glucose, and Oxygen Concentration on the Metabolism and Proliferation of Rabbit Adipose-Derived Stem Cells

K.E. FOLLMAR, B.A.,[1] F.C. DECROOS, M.D.,[1] H.L. PRICHARD, B.S.,[2] H.T. WANG, M.D.,[3] D. ERDMANN, M.D., Ph.D.,[3] and K.C. OLBRICH, Ph.D.[3]

ABSTRACT

The use of adipose-derived stem cells (ASCs) for tissue engineering involves exposing them to metabolically adverse conditions. This study examined the metabolism, proliferation, and viability of ASCs under various oxygen, glucose, and glutamine concentrations to determine how these cells respond to such environments. ASCs were cultured in each of 8 media preparations containing 4.8 or 21.5 mM glucose, and 0, 2, 4, or 6 mM glutamine. The ASCs were cultured under normoxic (20% O_2) and hypoxic (0.1% O_2) conditions. Conditioned media were collected and assayed for glucose, glutamine, lactate, pyruvate, and glutamate. Cell proliferation and cell death were measured after 5 days of culture. ASCs remained metabolically active under all culture conditions; however, their proliferation rate was significantly reduced in the absence of glutamine. Hypoxia resulted in increased cell death. ASCs are a viable source of stem cells for tissue engineering purposes, although substantial challenges remain. These cells are able to survive in environments with limited oxygen and glutamine and thus may be able to survive brief periods of limited nutrient transport after implantation.

INTRODUCTION

ADIPOSE-DERIVED STEM CELLS (ASCs) have multipotent differentiation potential[1] and are thought to be a promising source of cells for a variety of tissue engineering applications.[2,3] These cells have also been used clinically in at least one case to promote osteogenesis in the repair of a pediatric calvarial defect,[4] and are also being used in a phase I clinical trial in the treatment of Crohn's fistulas.[5]

Maintaining the viability of cells after implantation may prove to be a significant barrier to the use of these cells for tissue engineering applications. It is expected that the microenvironment of implanted cells will have limited oxygen and nutrient transport. This poor mass transport environment may have long-term, detrimental effects on the survival, function, and differentiation of implanted cells.

Most studies on the characterization, proliferation,[6,7] and differentiation[8,9] of ASCs have been performed at atmospheric oxygen concentrations and in standard cell culture media. However, the conditions that ASCs encounter in cell culture experiments, where oxygen, glucose, and most other nutrients are plentiful, are very different from those that they are exposed to *in vivo*. When implanted in engineered tissues lacking a preexisting blood supply, ASCs are likely to encounter an environment in which molecular transport is limited by diffusion. Once the implanted cells deplete all the glucose and other nutrients immediately available to them, they will face a nutrient-poor environment high in lactate and other cellular waste products. This microenvironment is likely to affect their viability, function, and differentiation.

The effect of adverse metabolic conditions on ASC function and proliferation has been investigated in at least one

[1]Duke University School of Medicine, Durham, North Carolina.
[2]Department of Biomedical Engineering, Duke University, Durham, North Carolina.
[3]Division of Plastic, Reconstructive, Maxillofacial, and Oral Surgery, Duke University, Durham, North Carolina.

unique case. Under chondrogenic differentiation conditions, Wang *et al.* have shown that "low oxygen tension significantly inhibit[s] the proliferation of [ASCs], but induce[s] a two-fold increase in the rate of protein synthesis and a three-fold increase in total collagen synthesis [and] increase[s] glycosaminoglycan synthesis at certain time-points".[10]

The goal of this study was to evaluate the metabolism and proliferation of ASCs under culture conditions that mimic certain aspects of the *in vivo* microenvironment. Oxygen, glucose, and glutamine were chosen as the three metabolic parameters to vary in our experiments. Oxygen was chosen because it is perhaps the most obvious and most important molecule that cells lack when implanted into a living system. If ASCs are not able to survive for an extended period in relatively extreme hypoxia, they are only of limited use for tissue engineering applications.

Glucose plays a central role as an energy source in cellular metabolism; it is the primary source of ATP for cells utilizing either oxidative phosphorylation or anaerobic glycolysis. Glutamine is another source of metabolic energy for cells. It feeds directly into the Krebs cycle through the process of glutaminolysis. Glutamine is also an important building block for protein production and a variety of other cellular processes. Under some conditions, glutaminolysis can contribute significantly to the cellular metabolism.[11,12]

MATERIALS AND METHODS

Isolation and culture of ASCs

ASCs were isolated by the method described by Zuk *et al.*[1] Inguinal fat was surgically collected under sterile conditions from a 9-month-old female New Zealand white rabbit anesthetized with ketamine and xylazine. The harvested fat was washed with phosphate-buffered saline (PBS) and minced with a no. 10 blade. The minced fat was incubated in a solution of collagenase (1 mg/g of fat) in Hank's balanced salt solution (HBSS), at 37°C for 60 min while stirring. The lipid layer was aspirated and the cells were pelleted via centrifugation. The cell pellet was resuspended in red blood cell lysis buffer for approximately 15 s and the cells were again pelleted by centrifugation.

The pellet was resuspended in control medium consisting of low-glucose Dulbecco's modified Eagle's medium (LG-DMEM) + 10% fetal bovine serum (FBS, Gibco cat no. 16140-071, lot no. 1256405) + 1% penstrep (10,000 units/mL of penicillin, 10,000 μg/mL of streptomycin) and plated onto cell culture flasks. The cells were cultured at 37°C in 5% CO_2, and the media was changed every 48 h until day 5, when the cells were found to be confluent. The cells were lifted with 0.25% trypsin, and resuspended in a freezing solution containing 70% LG-DMEM, 20% FBS, and 10% dimethyl sulfoxide (DMSO). This suspension was frozen at –80°C.

Confirmation of differentiation potential

To verify that the isolated cells were multipotent, they were differentiated into bone and fat using previously described differentiation protocols. Briefly stated, cells were thawed, passaged, plated, and exposed to control media (PM-1), adipogenic differentiation media (DM-2), or osteogenic differentiation media (osteoblast media, Zen-Bio, RTP, NC). The use of the adipogenic differentiation media was halted after 7 days, and replaced with the preadipocytes media as recommended by the manufacturer. After 24 days of regular media changes, cells exposed to control media and cells exposed to adipogenic media were stained with oil red O. Osteogenic differentiation was confirmed via alkaline phosphatase activity (EnzChek Phosphatase Assay Kit, Molecular Probes, Eugene, OR).

Preparation of media

Eight media preparations were made, each containing either low glucose (LG) or high glucose (HG), and 0, 2, 4, or 6 mM L-glutamine . DMEM with 1000 mg/L of glucose, no glutamine, and no phenol red (Sigma cat. no. D5921) was used as the base media. The 4 LG media were prepared by combining 43 μL of DMEM; 5 mL of FBS; 500 μL of penstrep; 0, 500, 1000, or 1500 μL 200 mM L-glutamine in 0.85% NaCl; and 1500, 1000, 500, or 0 μL of 0.9% NaCl. For the HG preparations, DMEM with 4500 mg/L of glucose, no glutamine, and no phenol red was prepared by adding 3.5 g/L of D-glucose to the low-glucose base media. The 4 HG media were then prepared by combining 43 mL of this HG-DMEM with the same quantities of FBS, penstrep, L-glutamine, and NaCl as were used in the LG preparations. Because of the presence of endogenous L-glutamine in the FBS component, the "0 mM L-glutamine" media contained approximately 0.1 mM of glutamine. Assays for glucose, glutamate, lactate, pyruvate, and glutamine (described below) showed the contribution of each of these metabolites by FBS to be minor in all other cases.

Metabolic profiling experiment

Frozen ASCs were thawed, resuspended in control medium, plated onto cell culture flasks, and cultured to confluence at 37°C with media changes every 48 h. These ASCs were replated onto Lumox gas permeable 24-well plates (Grenier Bio-One) at a density of 50,000 cells/well.

Thirty-six hours after the cells were plated, the control media was aspirated from each well, and was replaced with 400 μL of one of the 8 media preparations. Each medium preparation was placed in 6 replicate wells containing cells in each of 2 plates (1 plate to be exposed to hypoxia and one to normoxia). In addition, each medium preparation was placed in 3 control wells without cells in each of 2 plates. These wells without cells served to control for the effects of evaporation on the media's composition.

One set of plates was placed in a hypoxia chamber containing, 5% CO_2, 0.1% O_2, and the balance N_2 (ProOx 110, Biospherix, Redfield, NY); the other set of plates was placed in a normal incubator chamber containing 5% CO_2 and 95% room air (~20% O_2). Both chambers were maintained at 37°C.

After incubating for 24 h, the conditioned media was aspirated, discarded, and replaced with 400 μL of fresh media of the same preparation. The plates were then returned to their hypoxia and normoxia chambers. Twenty-four hours later, the conditioned media were aspirated and stored for metabolite analysis. Fresh media (preequilibrated to the appropriate oxygen concentration) were added to the wells, and the plates were returned to their hypoxia and normoxia chambers. After an additional 24 h, the conditioned media were collected and saved for metabolic analysis. The naïve medium in each of the control plates (no cells) was collected, saved, and replaced in the same fashion as the conditioned media.

Each media sample was filtered with a centrifugal filter (MWCO 10K, Pall Life Sciences, East Hills, NY) to remove large macromolecules that may damage the microdialysis analyzer or interfere with the assays.

The concentrations of glucose, glutamine, lactate, pyruvate, and glutamate were measured for each of the 288 samples of naïve and conditioned media using a CMA 600 Microdialysis Analyzer (CMA, Solna, Sweden). The concentration of L-glutamine in each sample was measured by enzymatic conversion to γ-glutamylhydroxamate followed by colorimetric detection of γ-glutamylhydroxamate complexed with iron.[13]

Proliferation experiment

Frozen ASCs were thawed, plated, and cultured as previously described. First passage ASCs were plated onto 5 separate Lumox gas permeable 24-well plates at a density of 10,000 cells/well and incubated at 37°C for 24 h under normoxic conditions. The media were aspirated from all 5 plates. One plate was rinsed with PBS and frozen at –80°C to serve as an initial cell count. The remaining four plates were each divided into 8 sets of 3 wells, and each set was filled with 400 μL of 1 of the 8 media preparations. Two of these plates were returned to the normoxia chamber and the other 2 were placed in the hypoxia chamber at 0.1% oxygen. The medium in each well was aspirated and replaced with fresh medium every 24 h for 5 days. The first 4 days of conditioned media were disposed of, and the fifth day's conditioned media samples were frozen and later assayed for LDH activity.

After 5 days of media changes, one plate from each of the two chambers was rinsed with PBS and frozen at –80°C. All frozen plates were thawed, and the number of cells in each well was measured using a nucleic acid–based cell proliferation assay (CyQUANT® Cell Proliferation Assay Kit, Molecular Probes, Eugene, OR).

The cells in the remaining two plates were lysed in a total volume of 400 μL of lysis buffer and assayed for lactate dehydrogenase (LDH) activity using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI).

Data analysis and statistics

The glucose and glutamine consumption rates of the cells were determined by subtracting the concentration of metabolite in conditioned media from the average concentration in 3 samples of naïve media (no cells, but otherwise handled identically) and dividing by the incubation period of 1 day. The lactate production rate for sample was determined by subtracting the lactate concentration in naïve media from that in conditioned media and dividing by the incubation period.

The concentration of pyruvate and glutamate (micromolar) in media is significantly lower than are the other 3 metabolites (millimolar). Because these metabolites are actively transported across the cell membrane and can be both consumed and produced in different stages of the Krebs cycle, the final concentration is a function of the balance between production and consumption. Consequently, the final concentration is a better indicator of the metabolic state than the production or consumption rate. Therefore, only absolute final concentrations were reported. The concentrations of these metabolites in naïve media are also given as a reference.

Percentage cell death (or cell lysis) for each set of conditions was determined by dividing the LDH activity of the conditioned media (background corrected to naïve media) by the sum of the LDH activity of the lysate and that of the conditioned media.

Statistical analyses of the data were conducted using ANOVA with JMP 5.01 (SAS, Cary, NC). Dunnett's *post hoc* means comparison was used to determine individual means differences against the appropriate control group. Differences were deemed to be statistically significant when $p < 0.05$.

RESULTS

Confirmation of differentiation potential

Cells exposed to adipogenic media had significant accumulation of lipid droplets one week following initiation of differentiation, compared to no staining of cells grown in control media or osteogenic media (Fig. 1). After 6 weeks of differentiation, ASCs exposed to osteogenic media had alkaline phosphatase activity 2.01 ± 0.26 times greater than those in control media ($p < 0.05$).

Glucose metabolism

ASCs consumed glucose under all conditions (Fig. 2). Consumption was approximately 3–6 mM glucose per well

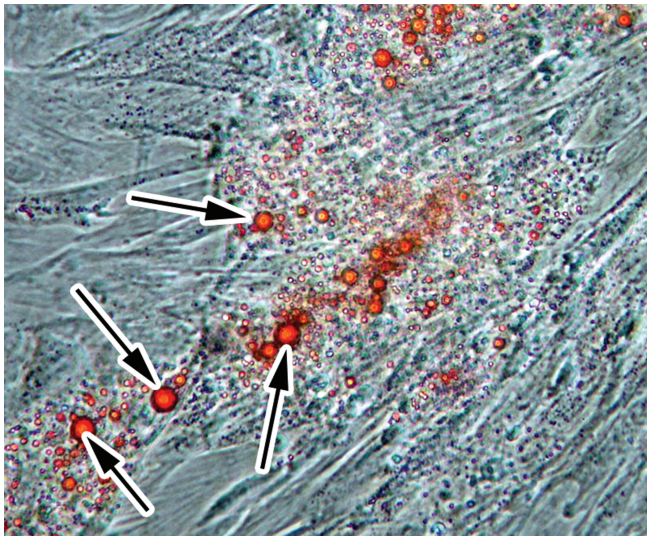

FIG. 1. Adipose differentiation of ASCs. Light micrograph of rabbit ASCs after 5 days of differentiation into adipocytes. Cells were stained with oil red O to accentuate lipid droplets (arrows). Cells treated with osteogenic or control media do not demonstrate lipid droplet formation (data not shown). Color images available online at www.liebertpub.com/ten.

per day. Glucose consumption was lowest in the absence of glutamine, and showed no significant change between 2 mM and 6 mM glutamine. There was a small, but statistically significant, increase in glucose consumption under hypoxic conditions and in the high-glucose media.

Glutamine metabolism

ASCs consumed a significant amount of glutamine under most conditions tested (Fig. 3). Oxygen and glutamine concentration had a significant ($p < 0.05$) effect on glutamine consumption, but glucose concentration did not. Glutamine consumption was significantly reduced under hypoxic conditions, and glutamine consumption increased with increasing glutamine concentration until 4 mM. There was no significant change in glutamine consumption between 4 and 6 mM.

Lactate metabolism

ASCs produced significant amounts of lactate under all conditions tested (Fig. 4). Lactate production increased a small, but significant amount between the low- and high-glucose media. Lactate production also increased significantly under hypoxic conditions, and in the presence of glutamine. It was also noted that lactate production was strongly inhibited in the absence of glutamine under normoxic conditions. Under hypoxic conditions, this inhibitory effect was absent. Lactate production showed a small increase with increasing glutamine concentration over the entire concentration range.

Pyruvate metabolism

The pyruvate concentration of conditioned media (Fig. 5) was influenced by the concentrations of glutamine and oxygen. Glutamine concentration did not play a large role in controlling pyruvate concentration with the exception that the pyruvate concentration was quite low in the absence of glutamine under normoxic conditions Hypoxic conditions significantly decreased the pyruvate concentration in the media and simultaneously eliminated the decrease in pyruvate concentration observed in the absence of glutamine under normoxic conditions.

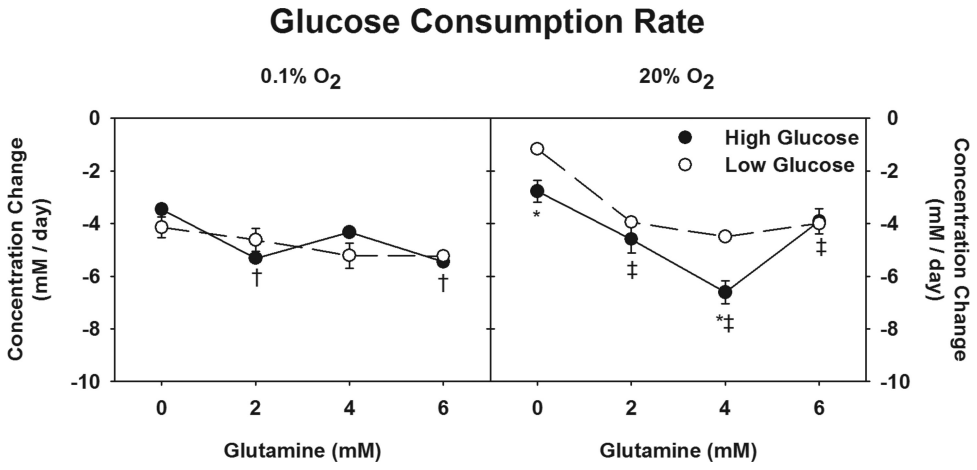

FIG. 2. Glucose consumption. Under hypoxic culture conditions, the glucose consumption rate of rabbit ASCs was not significantly different between the high- and low-glucose media. Under normoxic conditions, the glucose consumption rate increased significantly ($p < 0.05$) with glutamine concentration, and was significantly higher for ASCs in high-glucose media than for those in low-glucose media ($p < 0.05$). †Cells in high-glucose significantly different from no-glutamine condition ($p < 0.05$). ‡Cells in high- and low-glucose significantly different from no-glutamine condition ($p < 0.05$). *High-glucose significantly different from low-glucose condition ($p < 0.05$).

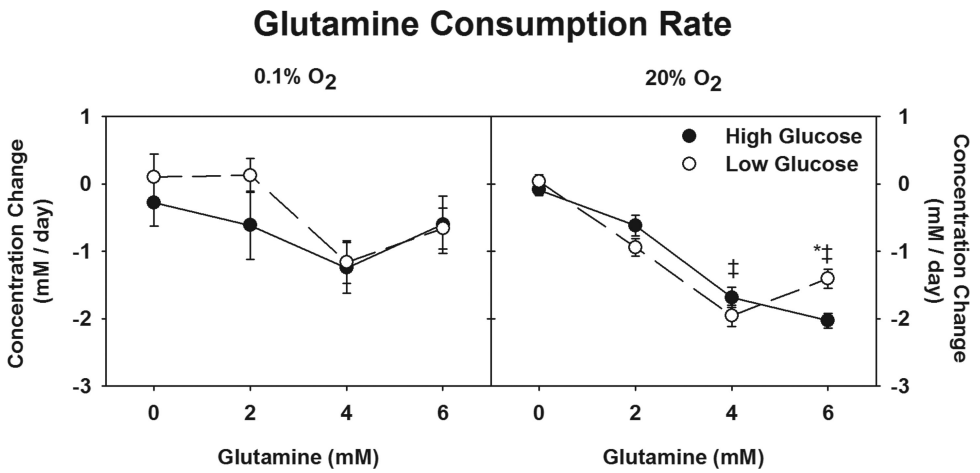

FIG. 3. Glutamine consumption. Glutamine consumption under normoxic culture conditions increased significantly ($p < 0.05$) with increasing glutamine content in the media. The consumption rate decreased slightly at the highest tested glutamine concentration in low-glucose media ($p < 0.05$). Similar trends were observed under hypoxic conditions. The glutamine consumption rate was not significantly altered by glucose concentration. ‡Cells in high- and low-glucose significantly different from no-glutamine condition ($p < 0.05$). *High-glucose significantly different from low-glucose condition ($p < 0.05$).

Glutamate metabolism

The glutamate concentration of conditioned media (Fig. 6); was influenced by the concentrations of glutamine and oxygen and glucose. The concentration of glutamate was significantly higher in the high-glucose media than the low-glucose media. The glutamate concentration also decreased when cells were exposed to hypoxic conditions, and in media with no glutamine.

Cell proliferation

Total cell count increased over the 5-day period for all culture conditions tested (Fig. 7). Glucose, glutamine, and oxygen concentrations all had significant effects on the final cell counts. Proliferation of ASCs was significantly higher in high-glucose media than in low-glucose media. The proliferation of ASCs was also significantly inhibited by hypoxic conditions. The absence of glutamine nearly eliminated

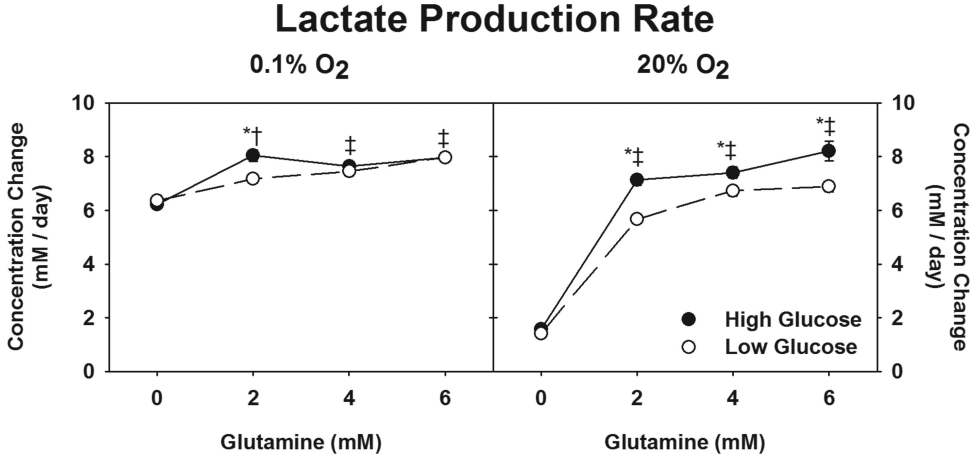

FIG. 4. Lactate production. Lactate production under hypoxic conditions was not affected by glucose concentration in the media. A small, but significant ($p < 0.05$), increase in the production rate was observed with increasing media glutamine concentration. Under normoxic conditions, lactate production was significantly lower ($p < 0.05$) in the absence of glutamine, and lactate production was also observed to be lower in low-glucose media ($p < 0.05$). †Cells in high-glucose significantly different from no-glutamine condition ($p < 0.05$). ‡Cells in high- and low-glucose significantly different from no-glutamine condition ($p < 0.05$). *High-glucose significantly different from low-glucose condition ($p < 0.05$).

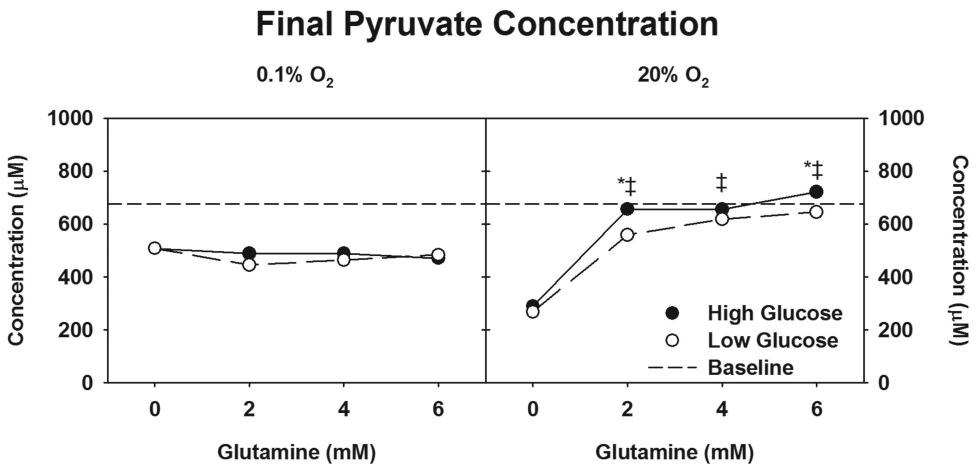

FIG. 5. Final pyruvate concentration. The final concentration of pyruvate in media conditioned by ASCs in hypoxic conditions was significantly lower ($p < 0.05$) than that found in baseline media (dashed reference line). Glucose and glutamine concentration had no effect on the pyruvate concentration. Under normoxic culture conditions, the conditioned media are not significantly different from the basal media in high-glucose media, and are slightly lower in low-glucose media ($p < 0.05$). A substantial and significant reduction was observed for cells in media with no glutamine under normoxic conditions ($p < 0.05$). ‡Cells in high- and low-glucose significantly different from no-glutamine conditions ($p < 0.05$). *High-glucose significantly different from low-glucose condition ($p < 0.05$).

cellular proliferation. Cell proliferation was greatest in 2 mM glutamine; higher levels of glutamine inhibited proliferation. High levels of glutamine in low-glucose media also inhibited proliferation of ASCs.

Cell death

The amount of cell death detected (Fig. 8) was significantly increased by hypoxia and low-glucose media. The glutamine concentration of the media had no significant effect on cell death; even at the lowest (0 mM) and highest (6 mM) tested concentrations.

DISCUSSION

The effective use ASCs for tissue engineering applications requires knowledge of how these cells respond to environments with limited nutrient availability. This study

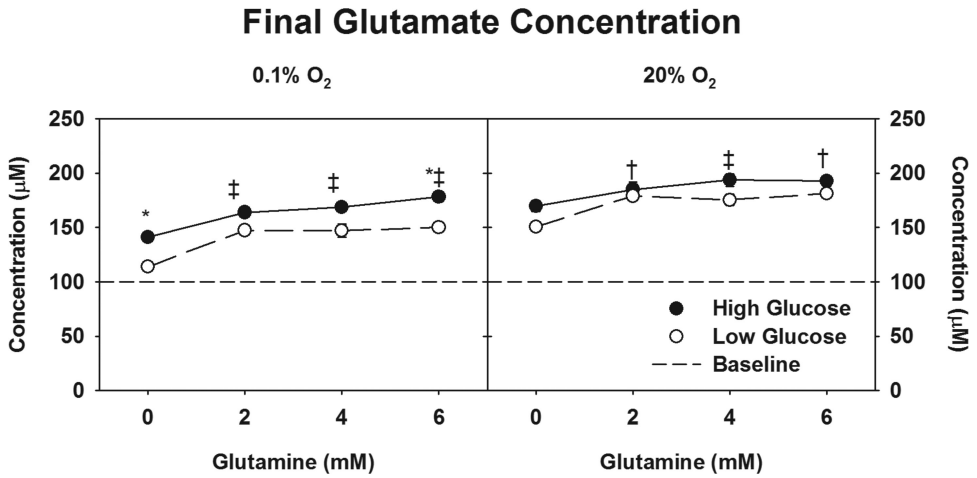

FIG. 6. Final glutamate concentration. The final glutamate concentration in conditioned media was significantly ($p < 0.05$) elevated above levels normally found in basal media (dashed reference line). The glutamate concentration was significantly ($p < 0.05$) higher in high-glucose than in low-glucose media. Glutamate levels also demonstrated a small, but significant ($p < 0.05$) difference between normoxic and hypoxic conditions. †Cells significantly different from no-glutamine condition ($p < 0.05$). ‡Cells in high- and low-glucose significantly different from no-glutamine condition ($p < 0.05$). *High-glucose significantly different from low-glucose condition ($p < 0.05$).

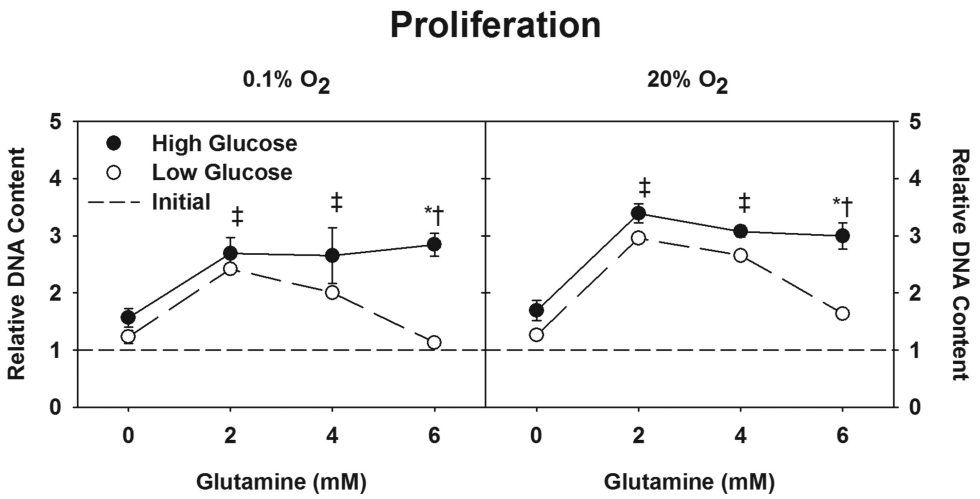

FIG. 7. Proliferation of ASCs. The values on this graph represent the ratio of the DNA content of cells after 5 days of culture normalized to the DNA content at the start of the experiment. ASC proliferation was significantly ($p < 0.05$) lower in media without glutamine, which nearly arrested cell growth. Proliferation was significantly ($p < 0.05$) higher in high-glucose than in low-glucose media under all conditions. ASC proliferation was significantly ($p < 0.05$) reduced at 6 mM glutamine for cells in low-glucose media. †Cells in high-glucose significantly different from no-glutamine condition ($p < 0.05$). ‡Cells in high- and low-glucose significantly different from no-glutamine condition ($p < 0.05$). *High-glucose significantly different from low-glucose condition ($p < 0.05$).

characterized the metabolism, proliferation, and death of ASCs under a variety of conditions. These data indicate that the metabolism of ASCs slows down in the presence of very low glutamine concentrations. Glucose consumption and lactate production were lowest in the absence of glutamine. While a small portion of this effect is likely due to decreased proliferation, this should have a minimal impact because these data were obtained 24–72 h after media stratification, leaving the cells relatively little time to proliferate.

Glucose consumption by ASCs was lowest under normoxic conditions in the absence of glutamine. This decrease in glucose consumption also correlates with the lowest rates of lactate production, glutamate and pyruvate concentrations, and cellular proliferation. The lack of proliferation without a simultaneous increase in cell death suggests an inhibition of proliferation rather than cytotoxicity due to a lack of glutamine. Yamauchi *et al.*[14] found that glutamine is required for the production of nucleotides. Without an adequate source of nucleotides, cells cannot produce DNA, which is essential for proliferation.

The low rates of glucose consumption and lactate production in the absence of glutamine suggests that these cells

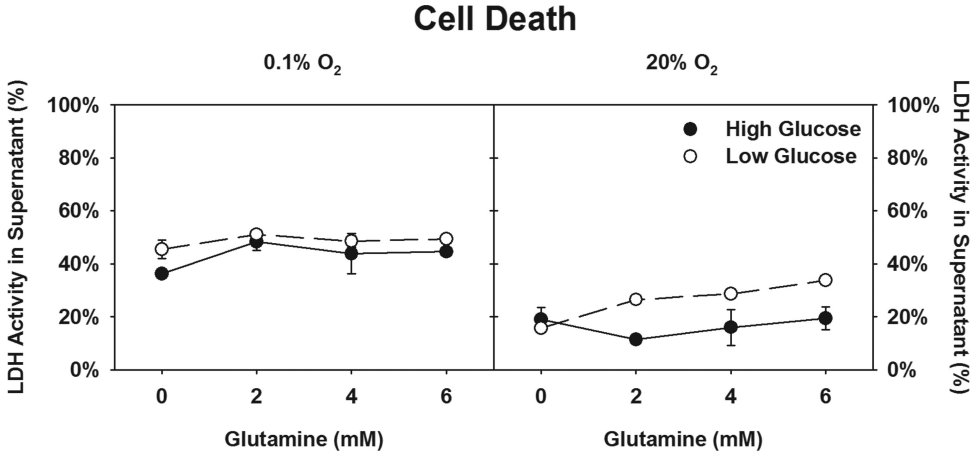

FIG. 8. Cell death. The fraction of lysed cells was determined by the fraction of total lactate dehydrogenase activity present in conditioned media after 5 days of culture. Cell lysis was significantly ($p < 0.05$) higher under hypoxic conditions when compared to normoxic conditions. No significant differences were observed between low- and high-glucose media under hypoxic conditions.

are quiescent. The cells are either not proliferating at all (i.e., they are arrested at a particular stage of the cell cycle), or they are proliferating very slowly. In either case, they appear to be consuming only enough glucose to maintain essential cellular functions.

The lactate production of the ASCs was substantial under all conditions in which cellular proliferation was observed. High rates of glycolysis under ostensibly aerobic conditions have been frequently observed in various cancer cell lines and also in normal proliferating vascular smooth muscle.[15] This elevated glycolytic rate may be a direct consequence of alterations in metabolism caused by cellular proliferation, or it may be a consequence of increased oxygen consumption by the ASCs. An elevated oxygen consumption rate driven by cellular proliferation may cause local hypoxia in the cells even under normoxic culture conditions. The cells may respond to this local hypoxia by relying more on glycolytic metabolism. Glucose consumption and lactate production increased under hypoxic conditions, which is consistent with a shift from oxidative phosphorylation to anaerobic glycolysis.

Relatively high levels of cell death were observed under hypoxic conditions. However, it is possible to culture these cells long-term (>21 days) under these conditions (data not shown). This suggests that a subpopulation of cells within the ASC preparation is susceptible to hypoxia-induced cell death or is dependent on aerobic metabolism. ASCs are known to be a heterogeneous mixture of cell types, and are not a uniform cell population.[16,17] The functional consequence of this alteration in population composition is unknown, as the precise identity of the susceptible cells is unknown. Immunologic-marker and functional studies are needed to determine which subpopulations of ASCs survive hypoxia-induced cell death, and whether the surviving cell population retains their capacity for differentiation.

CONCLUSIONS

In the absence of glutamine, ASCs adopt a quiescent-like metabolic state. While in this state, they consume little glucose, and cease proliferation. The ability to induce a temporary quiescent-like state in implanted cells may be useful for tissue engineering applications. Halting the proliferation of the implanted cells reduces their metabolic requirements, thereby reducing the flux of glucose, oxygen, and other nutrients required to maintain their viability. These reduced metabolic requirements may allow these cells to survive longer in a hostile microenvironment, which may allow angiogenic processes to establish an adequate nutrient supply before they succumb to nutrient deprivation.

In this study, elevated levels of cell death were observed under hypoxic conditions. The differentiation capacity of the hypoxia-sensitive subpopulation is unknown, but may play a significant role in the utility of ASCs for tissue engineering applications.

Despite the effect of hypoxia on ASCs, they are a robust population of cells capable of surviving and remaining metabolically active in a diverse range of conditions. Clearly, further research is required to understand how the differentiation capacity and viability of these cells are affected by their microenvironment. A thorough understanding of the interplay between cellular metabolism and the local microenvironment will aid in the rational design of engineered tissues.

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Address reprint requests to:
Kevin C. Olbrich, Ph.D.
Division of Plastic, Reconstructive, Maxillofacial, and Oral Surgery
Box 3906
Duke University Medical Center
Durham, NC 27710

E-mail: kevin.olbrich@gmail.com