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BIOLOGY

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In normal cells caloric restriction has been shown to reduce rates of apoptosis and increase replicative age. Anti-aging effects are linked to the up-regulation of SIRT1. Caloric restriction has also been shown to reduce the proliferation of cancerous cell lines; however, the regulation of *SIRT1* in cancer cells is not clear. In addition, the effects of specific dietary restriction is another area that has not been well studied. In this study, MCF-7 breast cancer cells were grown in media supplemented with varying amounts of glucose. Periods of cell growth in glucose depleted media began with 500,000 cells, after 10 days cell counts showed 1,984,000 cells for cells grown in 0.0g/L, 6,122,560 cells in 1.0g/L, and 12,693,120 cells in 4.5g/L (normal). Analysis of protein expression found *SIRT1* to be up regulated in MCF-7 cells; by day 10 of growth in glucose free media its down regulation was induced. The methylation of the *hTERT* promoter was also examined, but no change was seen in its methylation status. These results indicate glucose depletion may be a possible supplemental measure to reduce breast cancer progression.

INTRODUCTION

Caloric restriction has shown lifespan extension in organisms as diverse as yeasts, drosophila, spiders, and mice. It is the only dietary measure known that shows an increase in maximum lifespan instead of average lifespan alone. One gene found to be upregulated as a result of caloric restriction is Sir2, which was first identified in calorie restricted yeasts, an increase in its expression has been shown to promote longevity [1]. In mammals, its orthologe, *SIRT1*, a NAD-dependent deacetylase, has been shown to increase longevity and decrease rates of carcinogenesis. Its activation is thought to be part of a defense mechanism that promotes survival of the cell in response to stress by deacetlylating, and subsequently deactivating, tumor suppressor genes that lead to apoptosis of the cell [2,3]. However the regulation of *SIRT1* in cancer cells is not clear. Recent studies suggest the effects of its expression may vary with cell type [4,5]. Increased rates of *SIRT1* expression may also be a factor in carcinogenesis [10]. In one study silencing of *SIRT1* greatly increased rates of apoptosis in epithelial cancer, and had no effect on noncancerous cells [10]. This was done in the absence of applied stress suggesting that the function of *SIRT1* differs between cancerous and noncancerous cells [10].

Calorie restriction has been shown to effectively increase expression of the gene *SIRT1* [1]; however, the effect of specific types of dietary restriction has not been well studied. The identification of dietary measures that restrict specific caloric sources and induce the same effects as total caloric restriction would aid in the identification of the specific metabolic pathways involved. Studies in our lab have shown that the specific dietary restriction of glucose may induce the same response as calorie restriction. Expression of *SIRT1* was increased and a reduction in the replication rate of ageing in MRC-5 normal lung fibroblasts *in vitro* was seen with glucose depletion. Research on the effects of glucose depletion on the expression of *SIRT1* in cancer cell lines is needed to give useful comparative data.

For the development of effective treatments, it is important to understand the mechanisms that affect the expression of *SIRT1*. In this study the expression of *SIRT1* in MCF-7 breast cancer cells grown in glucose depleted media was examined. An examination of the patterns of *SIRT1* regulation is necessary to understand the effects of glucose restriction in normal and cancerous cell lines.

Epigenetic changes in the promoter region of *hTERT*, specifically methylation patterns, were also examined. Epigentic studies involve genomic modifications that do not affect the primary sequence. These factors have been shown to influence the regulation of genes associated with cancer and aging [6, 7]. Changes that occur in promoter regions affect the expression of specific genes. The regulation of *hTERT*, the catalytic subunit of telomerase is a useful marker for the effects of anti-cancerous treatments. Increased levels of telomerase are seen in most types of cancer and serve as a mechanism to escape cellular senescence by restoring telomere length [8]. In contradiction to the

idea that methylation decreases transcription, cancer cells that express *hTERT* are found to be hypermethylated [9].

A clearer understanding of the effects of treatments such as glucose restriction would lead to the development of practical treatments that would have the same benefits as CR. This could help identify the causal relationship between dietary alteration and ageing related to metabolic activity, and in addition lead to the development of alternative treatments that reduce the progression of cancer.

MATERIALS AND METHODS

Cell culture: The effects of glucose depletion was simulated with MCF-7 cells plated and grown in glucose free DMEM media supplemented with 10% FBS, APS, 25 mg/ml L-gutamine with the addition of 4.5 g/L (normal amount), 1.0 g/L, or 0.0 g/L of glucose. The cells were counted at 1, 4, 7 and 10 days on a hemocytometer using standard Trypan blue staining. DNA and proteins were extracted for analysis.

Analysis of protein expression: Changes in the expression of *SIRT1* (~120 kda) were visualized through immunoblot analysis for each day and glucose concentration. For SDS polyacrylamide gel electrophoresis 100 µg of protein (extracted with CHAPs lysis buffer) was boiled in an equal amount of 2X SDS loading buffer for 10 minutes. Marker and samples were loaded into wells of a 5% SDS polyacrylamide gel. The gel was electrophoresed at 135V until dye reached the bottom of the gel. Transfer of proteins was done at 100V for 60 min at 40°C. The membrane was blocked overnight in 5% milk and TBST at 40°C. The membrane was probed with *SIRT1* antibody with a 1/500 dilution in blocking buffer for 1 hr at room temperature. The membrane was washed 3X with TBST, 15 min per wash. The membrane was probed with secondary antibody for 1 hr sealed in parafilm. The bound antigens were visualized by enhanced chemiluminescence.

Analysis of epigenetic changes: Changes in methylation patterns were analyzed for each interval of cells grown in glucose depleted media (0.0g/L) and normal controls (4.5g/L). MethylEasy DNA bisulphite modification kit (Human Genetic Signatures, Macquarie Park, Australia) was used for identification of changes in methylation patterns. For each sample, up to 4g of DNA was mixed with NaOH solution then incubated at 37°C for 15 min. Bisulphite modification was performed at 55°C overnight to ensure complete conversion of nonmethylated cytosines. Samples were cycled through nested PCR for two rounds using primers F1 5' GTTTTTAGGGTTTT-TATATTATGG F1. 5' AACTAAAAAAAATAAAAAAA-CAAAAC R1. 5' GGGTTATTTTATAGTTTAGGT F2. 5' AATCCCCAATCCCTCC R2. Amplified DNA was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA); Amplified segments were sequenced using the 3730 DNA sequencer.

RESULTS AND CONCLUSIONS

Each period glucose depletion experiment began with 500,000 cells. By day 7 of the treatment, the cells treated with 4.5 g/L of glucose had a population of nearly a million more cells than those treated with 0.0 g/L of glucose (Figure 2). By day 10 the cells that were given no glucose finished with a population of 1,984,000. The cells with a decreased amount of glucose, 1.0 g/L, finished with a population of 6,122,560 cells, while the population treated with 4.5 g/L of glucose had 12,693,120 cells by the end of day 10 (Figure 2). While the three populations all began at nearly even cells counts on day 1, by the end of day 10 the cells grown in 4.5 g/L of glucose

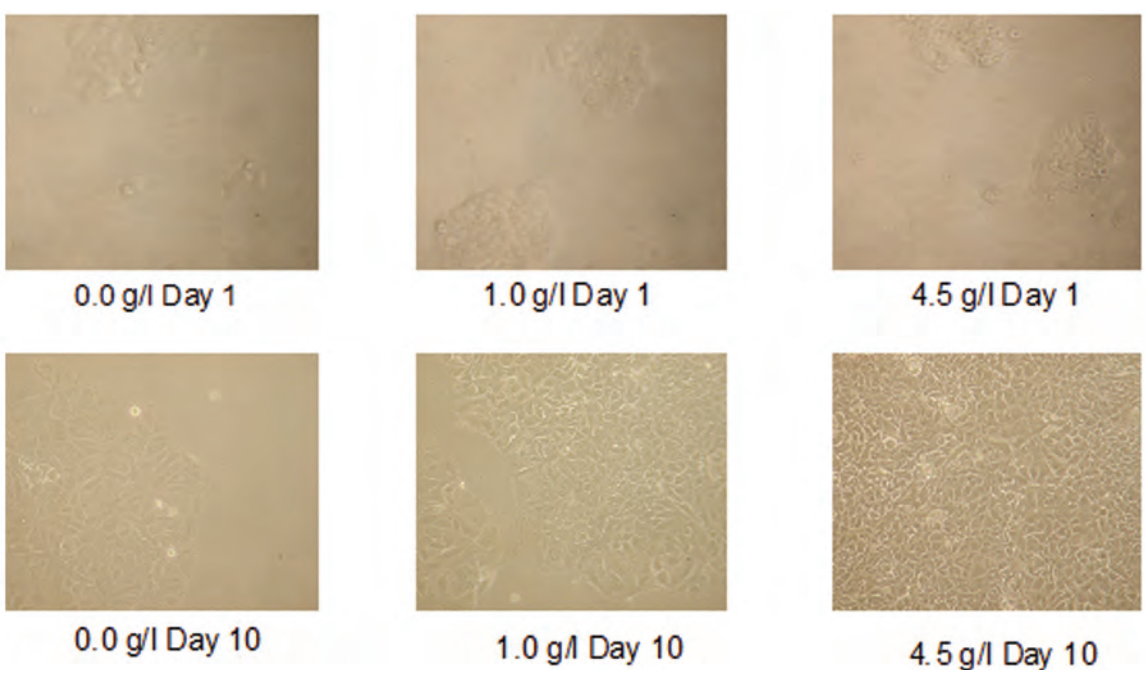

Figure 1. The MCF-7 breast cancer cells were grown with varying amounts of glucose in the medium. Pictures were taken every three days to track cell growth. The pictures for Day 1 and Day 10 for each concentration are shown.

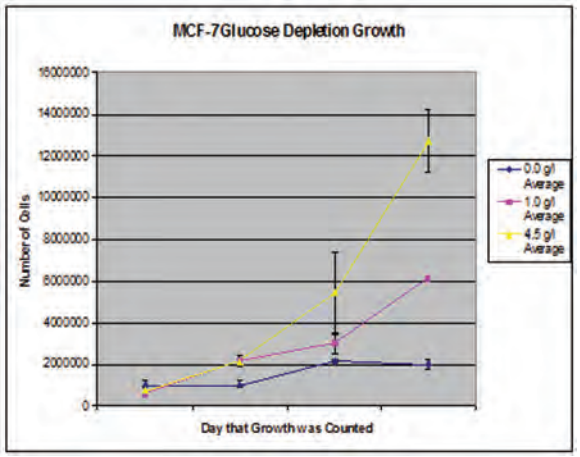

Figure 2. Growth rates for the MCF-7 breast cancer cells treated with varying concentrations of glucose in three day intervals: d1, d4, d7, and d10.

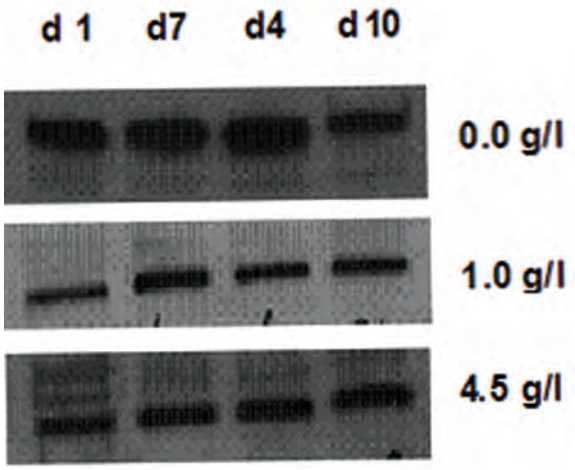

Figure 3. Western blot analysis of the expression of SIRT1 at three day intervals, for cells grown in varying concentrations of glucose.

were much more confluent than those grown in both 1.0 g/L and 0.0 g/L of glucose (Figure 1). These cell counts indicate that glucose depletion reduces the cellular proliferation of MCF-7 breast cancer cells.

Western blot analysis was done for days 1, 4, 7, and 10 of concentrations 0.0g/L, 1.0g/L and 4.5g/L. An increase in the expression of *SIRT1* was seen over the period of days 1 to 7 at 0.0g/L (Figure 3). At day 10 of this treatment a decrease in the expression of *SIRT1* was seen (Figure 3). For concentrations of 1.0g/L no discernible change in expression was seen over the duration of the treatment. The control concentration (4.5g/L) also showed no changes in expression. In conclusion, *SIRT1* was found to be over expressed in MCF-7 cells. By 10 days of growth in glucose free media its down regulation was induced. In a study by Ford et al. [10] *SIRT1* was found to enable the growth of human epithelial cancer combined with our results this suggests *SIRT1* may be a possible target for cancer therapy.

The promoter region of *hTERT* had 24 methylation sites present at day 1 for cells grown in glucose free media. Cells grown in normal media (4.5 g/L) also had 24 sites at day 1. From day 1 to day 10 no change was seen in the number of 5 -methylcytosines present in the *hTERT* promoter region for cells grown in glucose free media (Figure 4). At the end of the 10 day growth period both cells grown in glucose free media and those grown in the control had 24 methylation sites. No changes were found in the methylation status of the *hTERT* promoter after 10 days of growth in glucose free media. This indicates that *hTERT* is not down-regulated in glucose depleted cells by changes in the methylation patterns of its promoter. Future experiments could concentrate on histone acetylation analysis at *hTERT* promoter along with bisulphite sequencing and histone acetylation analysis of *SIRT1* promoter of MCF-7 breast cancer cells.

These results suggest that, in combination with traditional cancer therapies, glucose depletion may be an effective supplemental measure to reduce breast cancer progression. Future studies could investigate the effects of *in vivo* glucose depletion, in addition to studies that target depletion of other nutrients such as lipids.

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