Medium for Gene Therapy: Improved Protein-Free Media for Growth and Production of Viral Vectors for use in Gene Therapy

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Abstract

The heightened need for animal component-free protein-free media for the production of cell culture derived human therapeutic agents exists due to the potential presence of adventitious agents in the animal derived components commonly utilized in cell culture. This need is particularly apparent in the production of viral vectors for gene therapy applications since many adventitious agents would be expected to copurify with the vector. We report here a medium free of proteins and of animal derived components intended for use with the Per.C6® cell line for the production of adenoviral vectors. Through modification of the medium composition, we have been able to facilitate a metabolic shift of the cells, which increases the longevity of cultures without adverse effects on the rate of cell growth and density. In this medium (Sigma G9916), cells have been adapted to growth in spinner cultures and sub-cultured for a minimum of 20 population doublings without changes in population doubling times. No weaning was necessary; as cultures have been initiated from frozen, serum-supplemented stocks by direct transfer of cells into this medium. Cultures reached maximum cell density (5-6 x 10E6/mL) between days 5 and 6 when inoculated at 250,000 cells/mL. Cells grown in this protein-free medium have been successfully frozen in liquid nitrogen and recovered.

Introduction

There is a strong demand for improved technologies in the field of gene therapy. Many vectors in gene therapy are based on viruses, with adenoviruses being used as a common delivery system. However, the presence of wild type replication competent adenoviruses (RCAs) is a major problem for the application of these vectors in gene therapy. These virus vectors require modifications that will eliminate recombination of wild type adenovirus during their therapeutic use. The elimination of the potential generation of wild type RCAs can be achieved by deleting a section of the viral genome and placing a sequence in the helper cells that does not overlap the deleted viral segment eliminating the potential for homologous recombination between the vector and the helper cell sequences and the production of RCAs. Crucell (Leiden, The Netherlands) has created such a system to prevent the formation of RCAs. They developed a helper cell line called Per.C6® containing the adenovirus serotype 5 (Ad5) E1A- and E1B-encoding sequences (Ad nucleotides 459-3510). Combination of the Per.C6® cells and the nonoverlapping E1 deleted adenoviral vectors eliminates the issue of generating RCAs by homologous recombination, therefore producing safe recombinant adenovirus vectors. The increasing demands for Per.C6® cells and virus production in the industrial and pharmaceutical sectors has created the need for an animal component-free protein-free medium in which to produce the therapeutic vectors. Sigma has assisted in meeting the demands by improving the formulation of our serum-free suspension medium. Modification of the original medium has lead to an increase in cell density while supporting increased virus production.

Materials and Methods

Materials: Per.C6® cell stocks were obtained from Crucell (Leiden, The Netherlands). All materials used in this work were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Methods: Stock cultures of Per.C6® cells were grown in G9916 medium. The cells were passaged three times per week and seeded at 2.5 x 10^5 cells/ml. Cell cultures did not reach densities greater than 2 x 10^6 cells/ml. Cells were maintained in 1L spinner flasks (Techne, Inc., Princeton, New Jersey) on magnetic stir plates (Thermolyne Corp., Dubuque, Iowa) and incubated at 37°C and 5% CO_2.

Medium Development

The medium development process began by selecting several components of G0916 that were believed to have significant effects on cell growth. These significant effects were determined in previous assays. A growth assay was performed to identify the relative importance of the components in the medium and their effects on cell growth. The assay was set up by first preparing the test media with the necessary components and supplementing each medium with glutamine (4mM). One hundred ml of medium was added to each 100 ml spinner flask (Techne) and inoculated with 2.5 x 10^5 cells/ml. Spinner flasks were placed on stir plates in the incubator at 37°C and 5% CO_2 and stirred between 50-60 RPM. Total cell counts were done using the Schärfe CASY 1 TTC unit (Reutlingen, Germany) and viability was done by the trypan blue exclusion method. The concentration of viable cells/ml was then calculated as a percent. The spinner cultures were observed and counted daily until they declined to approximately 40% viable, at which point the spinners were terminated from the experiment.

After the components affecting cell growth and viability were identified, optimization assays were performed in order to determine the most favorable level of each desired component in the medium. The optimization assays were set up as described above, however, the selected components were tested at different concentrations in the medium.

Virus Production

Viral production experiments were set up in parallel with the cell growth assays. Single spinners were inoculated with Per.C6 cells® as above. After three days of growth at 37°C and 5% CO_2, the spinners were infected with 3 x 10^9 virus particles (moi≈3) of the rAd5 containing the ß-galactosidase (ß-gal) reporter gene (Crucell, Leiden, The Netherlands). The infected cells grew for an additional three days in order for viral replication to occur before collecting the virus for quantification. The collection process consisted of centrifuging each infected culture sample at 200g for five minutes. The supernatant was removed and the pellet was resuspended in 10mL of DPBS + 10% glycerol. Both supernatants and cell lysates were stored at -70°C. In preparation for quantifying the virus concentrations, a freeze/thaw process was performed three times to lyse the virus from the cells. The freeze/thaw process consisted of freezing the samples at -70°C and immediately thawing at 37°C. The samples were centrifuged at 200g for five minutes and the supernatant was drawn off for virus titration. In preparing for virus titrations, 96 well plates were set up by inoculating each well with 0.100 ml of attached Per.C6® cells (2.0 x 10^5 cells/ml) in DMEM (Sigma D5671) + 4mM glutamine + 10% fetal bovine serum (FBS) and incubated for three days at 37°C and 5%CO_2. When setting up the virus titrations, each cell lysate sample had six ten-fold serial dilutions from 10^{-5} to 10^{-10} done in DMEM (Sigma D5671) + 4mM glutamine + 10% FBS. Each dilution infected one column (eight wells) of a 96 well plate by adding 0.100 ml/well. A control column was set up with 0.100 ml of DMEM + 4mM glutamine + 10% FBS. The infected plates incubated for six days at 37°C and 5%CO_2. This 96 well plate design was set up for high throughput analysis for use with ß-gal assay.

Beta-galactosidase Assay

Detection of ß-gal activity was assayed using a Sigma ß-galactosidase Reporter Gene Activity Detection Kit (Sigma GAL-A). After the virus titrations in the 96 well plates finished incubating, the ß-gal colorimetric assay was performed. The medium was removed from the infected wells and 100ml of cell lysis buffer (1X) was added to each well. After a 20 minute incubation at 37°C, 100ml of ß-gal assay buffer (2X) was added to each well. The plates incubated for 30 minutes at 37°C. Next, a plate reader was used to determine absorbency values read at a wavelength of 405 nm. The absorbency readings were applied to the $TCID_{50}$ equation in order to obtain quantitative data.

Quantitation of Virus Production

The tissue culture infectious dose, 50 ($TCID_{50}$), a method for infectious virus quantitation, is defined as the dilution of sample at which 50% of the replicate cell culture inoculated with the sample becomes infected. The equation of Karber (1931) was used in calculating the $TCID_{50}$ value: -m= $\log_{10}$ starting dilution –[p-0.5] x d. The equation is defined where *m* is the $\log_{10}$ $TCID_{50}$ (per unit volume inoculated per replicate culture), *d* is the $\log_{10}$ dilution factor and *p* is the proportion of wells positive for viral infection. The number of positive wells came from the data obtained by the ß-gal assay. Positive wells are defined as having optical densities greater than 0.500.

Results and Discussion

Medium Development Study

The experimental assays were performed in order to understand which components in the medium were key to cell growth and viability. The components were identified and optimal levels of each component for the medium were determined. Based on the results of the optimization assays, three modifications were made to the original medium by changing the level of chelators, metals, and growth factors.

Comparison Study for Cell Growth and Virus Production

A study comparing Sigma's original G0916 medium to the newly formulated medium for production of virus was done. Cells were prepared and seeded in spinner flasks containing Sigma's original, modified medium and several commercially available media as described in the material and methods. The results shown in Figure 1 indicates cells grown in our modified medium (Sigma G9916) reached higher cell density (>4E6 viable cells/ml) than cells grown in any of the other media tested.

In order to confirm the modified medium formulation also supports adenovirus production, a comparison assay testing virus production in the same media tested for cell growth was performed. Following a standard system used in virus production as described in the material and methods, a ß-gal colorimetric assay was performed to assess viral productivity. As shown in Figure 2, three days post infection, the G9916 medium resulted in a virus titer of 1.50 x 10^9 particles/ml and the G0916 medium resulted in a virus titer of 5 x 10^8 particles/ml. These data suggest that Per.C6® cells produce more virus when growing in the modified medium (G9916) than in the original G0916.

Conclusion

Making the necessary modifications to G0916 medium resulted in a new animal component-free protein-free medium (Sigma G9916) formulated for production of adenoviral vectors in Per.C6® cells.

Producing a medium free from animal derived components supporting cell culture and adenoviral vector production will meet the needs of biotechnology and pharmaceutical manufacturers. When comparing the modified medium formulation (G9916) to the original G0916, the modification resulted in a metabolic shift in the cells in which initial growth was slower, but after six days of growth cell density was consistently >3 x 10^6. The cultures consistently grew to a density of four to five million cells/ml versus two to three million cells/ml in the original product. We also have observed consistent growth of cultures for greater than two weeks in the modified medium. Along with the increase in total cell density and cell longevity, we observed a viral production of 1.0 x 10^9 viral particles/ml at three days post-infection.

These experiments show the paired system of rAd5 vector and Per.C6® cells are supported by Sigma's G9916 cell culture medium and indicates the medium can be used in large scale applications.

References

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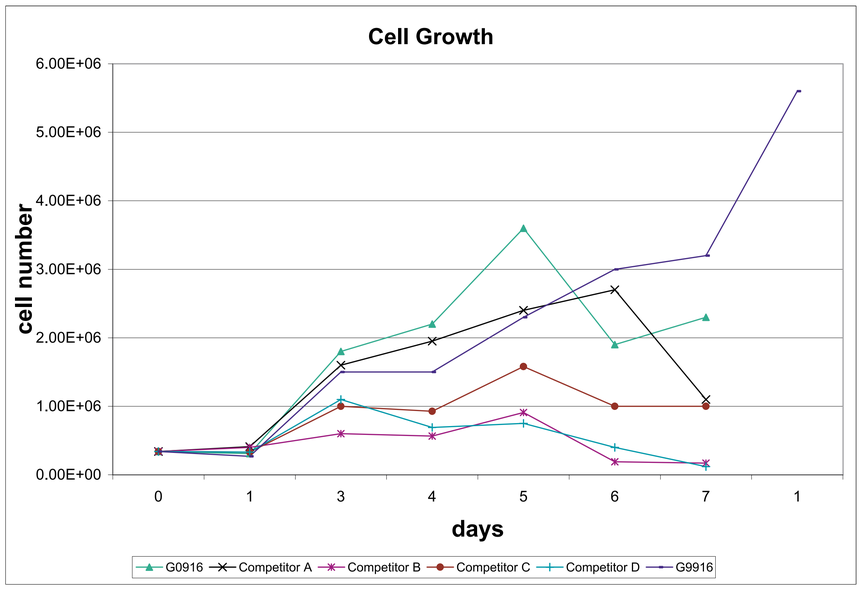

Figure 1. Comparison of cell growth of Per.C6® cells in G0916 medium and modified G0916 medium. Per.C6® cells were seeded at 2.5 x 10^5 cells/ml in spinner flasks with G0916 medium, modified G0916 medium (G9916) and several other commercially available media. Samples were taken on a daily basis in order to monitor cell growth and viability. Each data point represents the average viable cell number from duplicate spinner flasks from multiple experiments. Per.C6® cells showed the best cell growth in the G9916 medium with 6.83x10^6 at day nine of the culture. The Per.C6® cells growing in G0916 medium reached a maximum density of 4.16x10^6 at day five of the culture.

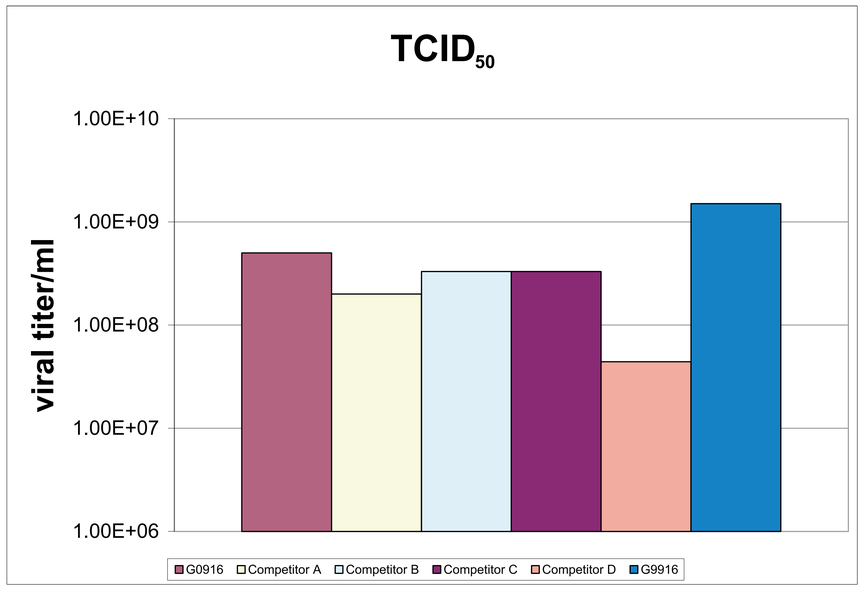

Figure 2. Comparison of recombinant adenovirus 5 in Per.C6® cells in Sigma's G0916 medium and modified G0916 medium. Per.C6® cells were seeded at 2.5x10^5 cells/ml in spinner flasks with G0916 medium, modified G0916 medium (G9916) and several other commercially available media. The cells incubated for 3 days at 37°C and 5% CO_2. Each flask was then infected with 3x10^9 virus particles. Samples were taken at three days post infection in order to analyze virus production. Sigma's G9916 medium supported the higher virus production in Per.C6® cells infected with the rAd5.