"GRAVITATIONAL EFFECTS ON SIGNAL TRANSDUCTION"

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ABSTRACT

An understanding of the mechanisms by which individual cells perceive gravity and how these cells transduce and respond to gravitational stimuli is critical for the development of long-term manned space flight experiments. We now propose to use a well-characterized model erythroid cell system and to investigate gravitational perturbations of its erythropoietin (Epo) signaling pathway and gene regulation. Cells will be grown at 1-G and in simulated microgravity in the NASA Rotating Wall Vessel bioreactor (RWV). Cell growth and differentiation, the Epo-receptor, the protein kinase C pathway to the c-myc gene, and the protein phosphatase pathway to the c-myb gene will be studied and evaluated as reporters of gravitational stimuli. The results of these experiments will have impact on the problems of 1) gravitational sensing by individual cells, and 2) the anemia of space flight. This ground-based study also will serve as a Space Station Development Study in gravitational effects on intracellular signal transduction.

TASK PROGRESS

METHODS

Cells. Rauscher murine erythroleukemia cells, a continuous cell line that differentiates in response to the hematopoietic growth factor erythropoietin (Epo), were grown in standard tissue culture dishes at unit gravity in a humidified atmosphere of 95% air/5% CO₂, 36.5 °C. The growth medium was Dulbecco’s modified Eagle medium, 10% fetal bovine serum. Cells were routinely subcultured every 2-3 days to maintain a cell count below 2 x 10⁶/ml.
Simulated microgravity. To generate a simulated microgravity environment in ground based experiments, we used the Rotating Cell Culture System (RCCS, Synthecon) equipped with a 50 ml disposable plastic vessel. This cell culture system is a modification of the original NASA Rotating Wall Vessel (RWV). It is characterized by a unique environment of low shear force, high mass transfer and suspended particle motion resulting in a constantly changing gravitational vector that simulates conditions found in microgravity. It has been used to culture several cell lines and tissues [1-15]. To initiate an experiment, cells growing in early log phase were harvested from their tissue culture flasks or dishes and were inoculated along with prewarmed complete medium into the RWV. Care was taken so that no bubbles remained in the chamber, which could greatly increase the shear and, thus, interfere with the simulated microgravity environment. The entire rotating chamber was placed in an incubator along with cells growing in flasks or dishes at unit gravity (1 x g). A rotation rate of 23 rpm was determined empirically to be ideal for the cells, keeping them in suspension while avoiding excessive centrifugal force. All sample additions to and removals from the RWV were made without stopping the rotation.

Cell growth and differentiation. Cells grown in tissue culture vessels or in the RWV were incubated in the absence or presence of 20 U of recombinant human erythropoietin (Epo)/ml for 0-4 days. At specified times, 1 ml of cell suspension was removed for cell counting using a hemacytometer and quantification of hemoglobinized cells by benzidine staining [16-18]. Removal of samples was accompanied by simultaneous injection of sufficient medium to displace the removed sample, thereby eliminating air introduction into the rotating chamber. To monitor growth conditions, daily glucose determinations were made using a glucose oxidase system (Stanbio Glucose LiquiColor). Standard curves were prepared using tissue culture medium as the vehicle in all experiments. Apoptosis was quantified by fluorescence microscopic identification of new 3'-OH DNA ends (ApopTag Plus™, Oncor).

RESULTS

Growth of Rauscher cells in simulated microgravity.

Ideal conditions for Rauscher cell growth in the RWV were determined empirically in a series of preliminary experiments. Cells were inoculated into the chamber at an initial concentration of 2 x 10⁵ cells per ml in complete medium.

In the absence of Epo (-Epo), cells grown in either dishes (1 x g) or the RWV exhibited log phase growth for 48-72 hours. Cells at 1 x g increased to 2 x 10⁶/ml in 48 hours (doubling time = 14.4 hours). In contrast, cells in simulated microgravity grew more slowly, achieving a concentration of 1 x 10⁶/ml in 48 hours (doubling time = 24 hours). Over 72 hours, cells at 1 x g grew at a rate of 1.1 x 10⁶ cells/day compared to 0.6 x 10⁶ cells/day in simulated microgravity. Similar results were observed in several repeat experiments. In the presence of Epo (+Epo), cells again exhibited log phase growth at 1 x g and in simulated microgravity. Again, the growth rate was greater at 1 x
g (doubling time = 15.6 hr) than in simulated microgravity (doubling time = 20.4 hr). The difference in doubling time could not be explained by a difference in apoptosis. At various time points at both 1 x g and in simulated microgravity, the number of apoptotic cells did not exceed 3-5%.

We compared glucose consumption under both growth conditions. Glucose consumption at 1 x g and at simulated microgravity were virtually identical over time. The glucose concentration of the medium decreased nearly linearly from 100mg/dl to 10mg/dl over 96 hours. When considered in light of the differing growth rates, however, glucose consumption per cell in simulated microgravity was approximately twice that observed at 1 x g. This observation remains unexplained. The absence or presence of Epo had no significant effect on glucose consumption. We determined that growth beyond the 72-hour time point resulted in glucose deprivation. Therefore, we elected to carry out experiments no longer than 72 hours. We carried out numerous repeat experiments like those shown above to ensure the reliability of the simulated microgravity model.

Effect of simulated microgravity on erythropoietin-induced differentiation

Having established optimal growth conditions for Rauscher cells in simulated microgravity, we carried out a series of experiments to assess their ability to differentiate in response to Epo and to compare this response with that of cells grown at 1 x g. We employed two strategies. The first began Epo treatment simultaneously with introduction of the cells into the RWV. The second began by “conditioning” the cells in the RWV for 24 hours. Then Epo was added and incubation was continued.

In the first type of experiment, Epo was added at time zero, along with the introduction of the cells into the 1 x g or simulated microgravity environment. Cells grown at 1 x g differentiated significantly with mean percent hemoglobin positive (Hb⁺) cells of 16%, 21% and 25% at 24, 48 and 72 hours, respectively. In contrast, under simulated microgravity conditions, differentiation was significantly lower with means of 8%, 13% and 12% Hb⁺ cells, respectively.

When the cells were “conditioned” by growing in simulated microgravity for 24 hours before the addition of Epo, this effect was even more striking. In the experiment shown, cells grown at 1 x g were 20% Hb⁺ after 48 hours of Epo treatment, a value similar to that observed in the previous experiment. However, the Hb⁺ percentage of simulated microgravity “conditioned” cells was markedly diminished, achieving a maximum of only 5%. This observation was highly repeatable. The maximal Hb⁺ cells obtained in three such experiments were 24%, 20% and 19% for cells at 1 x g, compared to 2%, 5% and 7% for cells in simulated microgravity. The inhibitory effect of simulated microgravity on erythroid cell growth and differentiation was reversible. After growth in the RWV for 24 hours, cells were removed and grown in tissue culture dishes in the absence or presence of Epo. Growth rates and differentiation were equivalent to those observed for cells that had not been grown in simulated microgravity.
Studies of the Erythropoietin Receptor

These results could reflect perturbations of the Epo/Epo receptor (EpoR) interaction. We have now begun to compare Epo/EpoR binding in cells grown in conventional tissue culture vessels with cells grown in the RWV using both Epo monomer and a bifunctional Epo-Epo fusion protein [19]. Using $^{125}$I-labeled Epo monomer, cells grown in flasks exhibited a single, high affinity class of binding sites. In contrast, the binding isotherm obtained using cells grown in the RWV was consistent with a two-site interaction, similar to the results obtained with the bifunctional Epo-Epo fusion protein on cells grown in flasks. A northern blot revealed an apparent increase of EpoR mRNA in cells grown in the RWV. Taken together, our results suggest both a change in expression of the EpoR gene and the appearance of a new protein-protein interaction in the plasma membrane involving the EpoR in cells grown in the RWV. We speculate that this new interaction could alter EpoR function and signaling efficiency.

REFERENCES CITED


**EARTH BENEFITS**

The results obtained thus far are consistent with the physiology of the anemia of space flight and support our proposed further studies of erythropoietin's signal transduction pathways. The anemia of space flight is a complex syndrome characterized
in part by a blunted response to erythropoietin resulting in reduced red blood cell production. This problem must be addressed. In addition, on Earth we see a similar blunted response in a variety of disease states, including the anemia found in cancer patients. It is possible that these two diverse conditions share some biochemical/molecular defects and that these defects in intracellular signaling can be modeled in the NASA RWV. Our further studies will dissect the signaling pathways triggered by erythropoietin and will identify those that are affected by microgravity. The results of this experimental approach could lead to new therapies for numerous anemic states, both on Earth and in space.

BIBLIOGRAPHY

Abstracts


Articles in peer reviewed journals


Articles in other journals (non peer reviewed)

Meeting papers / Proceedings


Academic dissertations or theses
Books or book chapters

Government Publications

Presentations / Lectures

Patents

No patents emanated from this award.

Awards

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