Texas Medical Center NASA/Johnson Space Center
Cooperative Agreement Program NCC 9-36, Round 1

FINAL REPORT

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Title: Utilization of microgravity bioreactor for differentiation and growth of human vascular endothelial cells

Goals of the Project:
The goal was to delineate mechanisms of genetic responses to angiogenic stimulation of human coronary arterial and dermal microvascular endothelial cells during exposure to microgravity. The NASA-designed rotating-wall vessel was used to create a three-dimensional culture environment with low shear-stress and microgravity simulating that in space.

The primary specific aim was to determine whether simulated microgravity enhances endothelial cell growth and whether the growth enhancement is associated by augmented expression of basic fibroblast growth factor (bFGF) and c-fos, an immediate early gene and component of the transcription factor AP-1.

Results:
The results of our experiments strongly support the hypothesis that simulated microgravity induces endothelial cell growth and expression of c-fos and bFGF. Most experiments were performed in bovine aortic endothelial cells (BAEC) because of the high grade homology between human and bovine bFGF and that between human and bovine c-fos. Unlike human cells, BAEC do not require high concentrations of serum or mitogens for survival and therefore provide an ideal model for investigating gene expressions.

To create simulated microgravity, cells mixed with microcarrier beads was cultured in the high aspect ratio vessel (HARV) system spinning around a horizontal axis (h-HARV; microgravity). Two types of control were used: 1) conventional monolayer culture without beads in T-150 flasks; 2) culture with identical cells and conditions except that the HARV device will be oriented to rotate around a vertical axis (v-HARV; near-ambient gravity). The estimated centrifugal force due to centrifugation in the v-HARV model was very small (14 RPM, radius 5cm, corresponding to 0.02G). Moreover, the cell-bead aggregates rotated at a rate less than 14 RPM such that centrifugal force was further decreased. For practical purpose, one can assume that cells cultured under v-HARV conditions were exposed to ambient gravity (~1G).

Cell replication.
Cell growth curves up to 168 hours were compared between h-HARV and monolayer cultures. The rate of proliferation and increases in actual cell counts in the h-HARV far exceeded those in monolayer cultures (Figure 1).
Figure 1. **Left Panel:** Proliferation rate of BAEC in h-HARV and T-150 at 0-168 hours. Note the most significant difference between 72 and 96 hours. At 120 hours, the cultures were confluent in both systems and contact inhibition occurred after that point. The overconfluency problem can be solved by increasing the microcarrier bead/cell ratio in h-HARV (data not shown). **Right Panel:** Actual cell count in h-HARV and T-150 at 0-168 hours. Note the great difference of cell numbers in the two systems.

In contrast to h-HARV, v-HARV failed to provide a stimulatory environment for the cells. Both attachment (to microcarrier beads) and actual cell numbers were below the values in h-HARV or monolayer cultures (Figure 2).

Figure 2. **Left Panel:** BAEC cultures at 120 hours (day 5) in h-HARV (left) and v-HARV (right). Note that all microcarrier beads in h-HARV were completely covered by cells, whereas most microcarrier beads in v-HARV were only one third to a half covered with cells. **Right Panel:** Proliferation rate of BAEC in h-HARV (h-H), v-HARV (v-H), and T-150 at 120 hours. Note the sharp difference between h-HARV and v-HARV, compatible with the findings shown on the left. The proliferation rate in T-150 was 340%, less than that of h-HARV (420%).
**Gene expression.**

Expressions of bFGF, c-fos, and a major bFGF receptor FGFR-1 were assessed both by reverse transcriptase-polymerase chain reaction (RT-PCR) and RNase protection assays.

c-fos expression was induced early in the incubation period, while bFGF expression was increased after 96 hours and remained elevated throughout the entire period of 168 hours. In contrast, expression of FGFR-1 was unchanged or slightly decreased.

**Figure 3.** bFGF mRNA levels at 96 hours quantitated by RNase protection assay. Note the slight increase (about 20%) in bFGF mRNA level in HARV (h-HARV) vs. monolayer (T-75) culture. The increment became larger after 120 hours (see Figure 4). All other RNase protection assays in progress are accompanied with β-actin controls as shown in Figure 1.

**Figure 4.** Effects of simulated microgravity on c-fos, bFGF, and FGFR-1 mRNA levels (detected by RT-PCR) in h-HARV (H) and monolayer controls (C) at 72, 96, 129, 144, and 168 hours. Note that c-fos increased at 72 hours and remained elevated throughout the course. bFGF increased slightly at 96 hours. At 120 hours, the increase in bFGF became obvious and was maintained throughout the course. In contrast, there was no change in FGFR-1, except for a reciprocal decrease when there was a marked increase in bFGF at 120 hours. β-actin was used for an internal control. M stands for marker.
Figure 5. Effects of simulated microgravity on c-fos, bFGF, and FGFR-1 mRNA levels (detected by RT-PCR) in h-HARV (H) and monolayer controls (C) at 24 hours. There was no change in bFGF or FGFR-1 levels. In contrast, c-fos increased at this early phase of experiment. This indicates that an incubation period of as early as 24 hours or even shorter can be used for a protocol for mechanistic investigation.

Cell cycle regulation.

The effects of simulated microgravity on cell cycle components were determined by flow cytometry (FCM). To analyze the G1, S, G2-M components during replication, a propidium iodide FCM staining procedure was used.

Cells removed from h-HARV, v-HARV, and T-150 monolayer cultured simultaneously for 5 days were resuspended, vortexed, and stained with propidium iodide (500 μg/mL). FCM analysis of the stained DNA, using the EPICS Profile I System (Coulter Inc.), was displayed (Figure 9).

Figure 6. FCM analysis of cell cycle progression in cells cultured in h-HARV, v-HARV, and T-150 for 120 hours.

In this example, most cells in both h-HARV and T-150 were in G1, with small proportions in S (4%) and G2+M (10-12%). In contrast, both S (21%) and G2+M (25%) were much higher in v-HARV. A possible explanation is that cells in both h-HARV and T-150 were already confluent and had reached a resting state, while cells in v-HARV were still proliferating.
Cell differentiation and angiogenesis.

It has not been reported whether cell differentiation and gene activation induced by microgravity can be preserved in daughter cells. In our preliminary experiments, we noted that 1st passage (now in monolayer) of cells from h-HARV (120 hours) remained polygonal with abundant cytoplasm (Figure 7, left panel). This particular pattern disappeared in the 2nd passage (data not shown).

![1st Passage from h-HARV](image1)

![Comparable Passage from T-150](image2)

**Figure 7.** Cell differentiation, monolayer cultures of 1st passages from h-HARV (left) and T-150 (right) cultured simultaneously for 120 hours. Note that cells from HARV were polygonal with abundant cytoplasm. The cells appeared healthy and were in confluency. In contrast, cells from T-150 were smaller and ellipsoidal in shape. There were more dead cells and the culture was still subconfluent.

Capillary-like microtube organization of the endothelial cells was facilitated in h-HARV and the life span of the microtubes was prolonged when macrophages were included in the co-culture.

![Capillary-like microtubes organized from endothelial cells co-cultured with macrophages after 9 days in the h-HARV](image3)

**Figure 8.** Capillary-like microtubes organized from endothelial cells co-cultured with macrophages after 9 days in the h-HARV.
Summary:
Our data provide strong evidence supporting our hypothesis that simulated microgravity facilitates endothelial cell growth and angiogenesis by inducing expression of selected genes. The results and an expanded theory can be summarized in a schematic figure (Figure 9).

Figure 9. Schematic summary of the hypothesis and preliminary findings.

Scientific Presentation:
Part of the findings reported here was presented at the NASA Biotechnology Investigators Working Group Meeting held in Houston, Texas, February 27-March 1, 1997. The presentation was well received.

Publication:
More data are being collected, even though the project has been officially ended, for the preparation of manuscript to be submitted to a highly ranked journal. The tentative title is:

“Stimulatory effects of simulated microgravity on endothelial expression of bFGF and c-fos”

The authors include Chen CH, Pellis NR, Luo S, and Henry PD.

Future plans:
1) Ground experiments: To determine the mechanisms (signaling pathways) of c-fos and bFGF induction in simulated microgravity and to seek clinical applications of the findings, e.g. stimulating angiogenesis for therapeutic purposes as in wound healing.
2) Spaceflight experiments: To verify the findings observed in the h-HARV cultures in true microgravity during spaceflight.

Would require further funding to support the experiments.
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Name of Project: Utilization of microgravity bioreactor for differentiation and growth of human vascular endothelial cells

Amount of Grant: (Total direct and indirect)

*Amount Spent, if Different from Amount Granted:

Date Project Was Completed: 6/30/97

Grants Officer: Thomas E. Wilson

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