SDS3/NASA 6 Mission
3-D Aggregation and Neuroendocrine Differentiation of PC12 Cells in Space

Peter I. Lelkes, PhD, Principal Investigator
Laboratory of Cell Biology , University of Wisconsin Medical School

Preliminary Report
May 10,1998

I Background and Hypothesis: Our long term goal is to use the microgravity environment, either simulated in Rotating Wall Vessel (RWV) Bioreactors or during space flight, to generated functional neuroendocrine 3-D constructs which may be useful as clinical replacement tissue in treating neurodegenerative diseases, such as Parkinson=s. Our previous ground-based studies have shown that neural crest-derived PC12 pheochromocytoma cells, a well established model for studying neuroendocrine differentiation undergo tissue-like assembly and neuroendocrine differentiation when cultured in RWV Bioreactors. As detailed in a recent publication (Lelkes et al, 1998, see appendix), we evaluated the effects of RWV culture conditions on the 3-D assembly, differentiation and function of rat adrenal medullary pheochromocytoma (PC12) cells. Using morphological, biochemical, and molecular biological assays we demonstrated that PC12 cells are exquisitely sensitive to the Asimulated microgravity@ environment in the RWV and yield macroscopic 3-D constructs in which the cells differentiate towards the neuroendocrine phenotype. ***In participating in this mission, we hypothesized, that simulated microgravity conditions in RWVs facilitate the formation of differentiated 3-D constructs of PC12 cells and that Atrue@ microgravity during space flight might enhance /accelerate phenomena observed in RWVs.***

II Objectives: The specific objectives of this mission (Bio 3-D on board STS86/MIR) were:
1. To optimize the experimental design for long-term cell culture in space under suboptimal conditions which accommodate the technical restraints/existing equipment on board MIR
- lack of conventional Acell culture facility@
- minimal maintenance
- limited post-experimental preservation (no cryogenic freezers, restricted choice of fixatives)

2. To test whether under these conditions cultured cells/cells assemblies can be passaged serially.

III Methods:

a) Cell Culture:

1. Inoculate 5 x 104/ml into Tissue Culture Bags (3 samples/time point)
_ Weekly sampling of Avital signs@ (glucose, pH, pO2, pCO2. HCO3- , electrolytes)
2. Passage at regular intervals (approx. q14)
_ remove 1/5, break up visible aggregates and inoculate into new bag
_ fix 4/5 (using *OmnifixTM*)

b) Sample Analysis:

1. <u>During Flight</u>: Macroscopic and microscopic observation/photography
2. <u>Post Flight:</u>
B Morphology: SEM, TEM, light microscopy
B Immunohistochemistry: Junctional Contacts, Adrenergic Differentiation
B Gene Expression by RT-PCR

IV Preliminary data from STS86: We participated, as guest investigators, in a long-term experiment (Bio3-D) on board Mir/STS86. The aim of this mission was to use PC12 cells as one of the cell types, previously proven to respond to the RWV culture conditions and to test the feasibility of long term culture and repeated (5 t o 6 times) passaging at ca.14 day intervals. Given the constraints on board Mir, both the cell culture conditions, and the long-term (up to 4 months) sample preservation/storage conditions were far from ideal. There were several major constraints on board Mir which impacted on the experimental set up for cell culture and sample retrieval/preservation: 1. The cells were maintained in 35 ml cell culture bags in an environment (inside the BSTC.), which provided for temperature control in an ambient gaseous environment (i.e. was not a regular CO_2 incubator); 2. No maintenance, besides periodic measurements of Avital signs@, i.e. dissolved gases (O_2, CO_2,-$COOH_3$) and pH was feasible, *viz.*, depleted nutrients could not be replaced, neither could pH or glucose be adjusted; 3. No cryogenic freezer for sample storage was available; and 4. Due to safety considerations on board MIR, no cross-linking fixatives, such as formaldehyde, could be used.

We decided to address these constraints by a) providing a highly buffered culture medium, b) inoculating the bags with a very low number of cells, which would allow for adequate growth without depleting nutrients, and c) preserving the cells at approx. 4^0 C in a fixative (from STRECK) which provided for adequate preservation of both structure and RNA. While the former goals were met, the latter aspect (cell preservation) was eventually compromised, due to an unforseen, last minute problem with the quality/consistency of the intended fixative. In choosing an alternative non cross-linking fixative (Omni-FIX), the ground tests (carried out over a two week period) proved to be fairly promising in that significant preservation of RNA was preserved. However, as our data showed, the longer the cells were exposed to this fixative, the more detrimental this Afixative@ was in terms of both loss of structural integrity and degradation of nucleic acids.

At this stage, some preliminary conclusions can be drawn based on the Avital@ signs (e.g. glucose consumption, pH etc., of the medium) collected during the flight, as well as our first morphological analysis of the samples themselves. Cell proliferation/metabolic status can be assessed from the rate of glucose consumption, as determined from periodic measurement of the glucose contents in the medium. As seen in **Figure 1**, glucose consumption (G) was fairly constant over time in the ground samples, while it increased with prolonged culture in space. The Arate of proliferation/metabolism@, plotted as the first derivative (dG/dt) of glucose consumption over time vs time, was significantly enhanced in space, as compared to the ground samples maintained under identical conditions. The higher rate of metabolism in space was also reflected by the faster ApH-consumption@, i.e., the rate by which pH in the medium declined to enhanced production of cellular waste products. As seen in **Figure 2**, the rate of pH consumption was significantly higher in space than on the ground. This difference is clearly seen when the slopes of the two previous curves are plotted for comparison.

The acceleration of the Awell being@of our cultures in space, as inferred from the enhanced Avital signs@, was also corroborated by the observations of Dr. David Wolf, the crew member responsible for carrying out the experiments on board Mir, who reported seeing and documenting very large aggregates in space. By contrast no such aggregates were observed on the ground. At the time of this writing (May 1998), the official macro- and -micro photographs taken by Dr. David Wolf during the experiments aboard MIR have not yet been released, but based on his preliminary reports, in each cycle of cell passaging, PC12 cells rapidly formed large, visible aggregates with the size of these aggregates growing with prolonged duration of the mission.

In spite of their compromised state of preservation, preliminary analysis of the samples strongly suggests the occurrence of enhanced 3-D aggregation in space. As seen in **Figure 3**, the space samples from both the first and last fixation time-points were found in spherical multicellular aggregates, which were significantly larger, than the clusters obtained in the ground-based control cultures. Preliminary

morphological analysis of the 3-D constructs by scanning electron microscopy suggested enhanced epitheloid, tissue-like differentiation of the cells comprising the aggregates in space. By contrast, PC12 cells in the space based 3-D constructs exhibit a distinctive, flattened epitheloid morphology with tighter cell-cell contacts than seen in the rather loosely assembled cells on the ground **(Figure 4)**.

Originally, we had planned to examine cellular differentiation by analyzing differential expression of neuroendocrine markers using reverse transcription-polymerase chain reaction-(RT-PCR) based assays. However, as already suggested by the disintegration of the aggregates and the accumulation of debris in the bags; nucleic acids were also significantly degraded, as confirmed upon isolation of RNA and DNA. (**Figure 5** and **Figure 6**). Consequently, we were unable to amplify any of the relevant RNA species, with the exception of the ubiquitous and abundant ribosomal RNA S28.

V Future analysis: We just received the remaining samples for purification and subsequent morphological analysis. In preliminary studies we plan to evaluate the (ultra) structure of the samples, depending on their quality, at the light and electron microscopic level. Also, we will conduct immunohistochemical studies to evaluate the expression/upregulation of specific markers and/or adhesion molecules involved in, respectively, organotypic adhesion and differentiation.

VI Preliminary conclusions: In spite of the sub-optimal experimental conditions on board Mir, these experiments clearly demonstrate that, a) repeated passaging of cells in space is feasible; and b) that the rate of cell growth/metabolism of PC12 cells is significantly (3-5 fold) accelerated in space as compared to ground. Thus, our first Aguest@ flight experiment partially fulfilled our initial scientific goals. In order to be able to publish our findings in peer-reviewed journals, these findings, have to be corroborated in future flight experiments In order to maximize the scientific return, culture conditions in space have to be systematically optimized. Based on our recent experience, we believe that the following issues that have to be addressed both on the ground during the flight definition phase and then implemented in space

1. Enhanced cell proliferation/metabolism requires stringent control of the culture environment, including regular monitoring of pH, gases, etc, concomitant with regular re-feeding schedules to maintain an adequate level of pH, glucose and nutrients. Also, given that our flight experiments were carried out under suboptimal conditions, during which at the end of each culture period both pH and glucose levels sank below acceptable norms, *we predict that optimized control culture conditions will yield even more dramatic results in terms of the accelerating effects of microgravity on cell growth/proliferation/metabolism and or differentiation.*

2. Given the real time link between the Shuttle/ISS and ground control, we anticipate to have a much better opportunity for optimizing culture conditions, by reacting immediately to any unforseen alteration in the cell culture environment (unexpected changes in temperature, pH etc.).We feel that the lack of such direct communication was one of the shortcomings of the last Mission.

3. In terms of the original goals: A*The BIO3D experiment will examine basic cell-to-cell interactions in a quiescent cell culture environment and investigate their role in the formation of functional tissue@* our experiment obviously fell short due to the above mentioned constraints. Specifically, the lack of adequate fixation/preservation prevented appropriate post-experimental functional analysis. Therefore, one of the major conclusions from our initial experiment is that besides establishing optimal culture conditions, a high priority has to be the use of reliable preservation techniques. Cryopreservation and/or use of proven cross-linking fixatives is essential in order to allow adequate analysis of the samples by morphological, immunological, as well as molecular biological techniques.

VII Figure Legends:

Figure 1: Comparison of glucose consumption in PC12 cells cultured on the ground and in space. For details, see text.

Figure 2: Comparison of pH consumption in PC12 cells cultured on the ground and in space. For details, see text

Figure 3: Morphology of 3-D PC12 cell constructs obtained on the ground (A,B) and in orbit (C,D). Fluorescence micorgraphs of representative samples fixed at the beginning and the end of the passaging study and stained upon return with the DNA stain Hoechst 22358

Figure 4: Scanning electron microscopic image of PC12 3-D constructs cultured in space and on the ground.

Figure 5: Long-term fixation in Omnifix caused degradation of total cellular DNA; Lanes 1,2: λ Phage DNA: 1: uncut; 2: cut 3-7: Ground Samples (dates of fixation) 3: 10/8; 4: 10/23; 5: 11/14 6: 11/20; 7: 11/29; 8-12:Space samples, dates of fixation as above DNA cut with HIND III: bands are 23 kb, 9 kb, 6 kb, 4.5kb, 2.2kb, 2.0kb and 0.5kb The dye fronts run at 50bp 300 bp and ca. 4 kb

Figure 6: PC12 cells 3-D constructs, grown in space and on the ground, were fixed on 11/20. RNA from these samples was isolated with RNAzol and assayed by RT-PCR. Probes used were for ribosomal S 28, TH and PNMT. Lanes 1,14: ctrl, Rat Adrenal, S 28 Lanes 2 -7: Ground Samples: 2,3:S 28; 4,5: TH; 6,7: PNMT; lanes 8-13: Space Samples, same as above

Bibliography:

Lelkes, P.I., Galvan, D.L., Hayman, G.T., Goodwin, T.J., Chatman, D.Y., Cherian, S., Garcia, R.M.G., and Unsworth, B.R. (1998) Simulated microgravity conditions enhance differentiation of cultured PC12 cells towards the neuroendocrine phenotype. In Vitro Cell Dev Biol, **34**:316-325.