HETEROGENEITY IN THE GROWTH HORMONE PITUITARY GLAND "SYSTEM" OF RATS AND HUMANS: IMPLICATIONS TO MICROGRAVITY BASED RESEARCH


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INTRODUCTION

Since its discovery over 50 years ago, growth hormone (GH) has attracted the attention of both clinical and basic researchers alike. The hormone accounts for 20% of the total protein contained in the anterior pituitary gland. Further, some 35-50% of the six different hormone producing cell types are committed specifically to the synthesis of GH. Milligram quantities of GH are present in the human pituitary gland whereas only microgram quantities of the five other peptide hormones are contained in this 500 mg, bean-shaped structure. Why should there be such a disproportionate amount of GH?

As the techniques to study cells and hormone molecules became more sensitive, it also became clear that heterogeneity was a landmark of pituitary GH. The picture that is emerging indicates that several variant forms of the hormone molecule exist within the gland as well as in the blood stream. New research also indicates that these variant forms possess unique biological activities. Finally, there is evidence to suggest that GH producing cells are also functionally heterogeneous. Our NASA sponsored research deals directly with these issues in both the human and rat pituitary gland. We believe that the large percentages of cells committed to GH production, taken together with the sizeable numbers of variant forms of GH, offer great flexibility to the "GH system". It is tempting to speculate that this flexibility helps the body meet the varied metabolic demands placed on it. Textbooks describe how GH increases muscle mass, increases breakdown of fat, decreases uptake of carbohydrate into cells and increases cell division. In light of these numerous activities, the recent claim that synthetic human GH "will soon be used to make short children taller and, possibly, help dieters lose fat and make aging people look young" (1) is therefore not surprising.

Spaceflight provides a unique environment which impacts the GH system on
several levels. First, GH controls muscle and bone metabolism, two tissues known to be negatively affected in flight. Second, cellular "life" as we know it has obviously evolved under the constant force of gravity. Evidence is beginning to accumulate that cell function, in the absence of gravity, is altered. Third, space bioprocessing takes advantage of the lack of convective forces in production of materials of medical relevance. Our NASA sponsored researches on GH touch on all three of these levels.

Before summarizing our experimental approaches and results, it is important to highlight the underlying theme of this work, viz. the importance of the assay system used to measure GH. Indeed, a dichotomy between bioassay-able and immunoassayable GH was recognized over 10 years ago (2). While the cellular and molecular basis for this dichotomy remain unresolved, our efforts show that it unquestionably exists. Our progress is summarized below.

I. Separation of GH cells.

The pituitary gland is markedly heterogeneous with respect to cell type. Over the last 20 years we have developed several methods to purify and study GH cells. These cell separation methods are now briefly reviewed. Special emphasis is placed on the different GH assay methods used to evaluate the performance of these cells once they are isolated.

A. Cell Separation by Sedimentation.

Our laboratory was the first to apply the techniques of velocity sedimentation at unit gravity and density gradient centrifugation to pituitary tissue (see (3) (4) for reviews). An important outgrowth of these experiments was the observation that two subpopulations of somatotrophs (GH cells) could be routinely separated on the basis of their differences in density (5). Key differences in cell morphology and cell function of these subpopulations are briefly summarized in Table 1. It is important to note that all of the GH
secretion data in that original study were obtained using an established GH radioimmunoassay. Can the dichotomy between immunoassayable and bioassayable GH, described above, be attributed to differential release of GH forms from Type I vs. II somatotrophs? Many experiments were done to test this possibility and the data in Fig. 1 suggest that this is indeed the case (6). In this experimental series, GH released from type I vs. II cells into tissue culture medium was assayed by standard immunological (I) procedures or by the rat tibial line bioassay (B). This bioassay, originally described in 1949, is based on the action of GH to promote widening of the tibial epiphyseal cartilage plate of hypophysectomized rats after eight injections over four days. Quantitation is accomplished by ocular micrometry of stained growth plates. While the procedure suffers from lack of sensitivity (5 ug), it is the accepted bioassay for GH. The data in Fig. 1 establish that GH initially released from type II somatotrophs has a much higher B/I ratio than type I cells.

It can be argued that the "ultimate" bioassay one could use to test the activity of GH released from type I vs. II somatotrophs would be their reimplantation into animals deficient in GH (i.e. a hypophysectomized rat). As we had developed the methodology to do this unique type of implantation experiment at about the same time the data in Fig. 1 were being collected, the experiment was relatively straightforward. The implantation method itself involves encapsulation of cells (usually 2.5x10^5 in 1.8 µl of culture medium) in 10 mm long XM-50 Amicon hollow fibers. These fibers are composed of a polyvinylchloride-acrylonitrile copolymer and have an internal diameter of either 500 or 1100 µm. Their unique feature is that the lumen of this fiber is lined with a thin membrane containing pores of well defined size, viz. 40Å (±5%). By virtue of this pore size, only spherical molecules <50,000 daltons
can theoretically pass through the membrane. Since GH has a mass of 22 kd, hormone released from encapsulated cells could in theory pass through the hollow fiber and enter the animals blood stream to deliver GH to its ultimate biological target(s).

Shown in Fig. 2 are micrographs of the hollow fiber as well as the appearance of cells within the fiber prior to implantation. The results of an experiment designed to test the biological responses of GH released from Type I vs. II somatotrophs in this hollow fiber system is offered in Fig. 3. These data show that animals implanted with type II somatotrophs a) gained more body weight, b) had increased tibial epiphyseal width and c) had increased muscle weight. Since the relative percentage increases in muscle mass were greater than increases in total body weight, we conclude that lean body mass was stimulated by hormone released from type II cells. We have seen this kind of result in several additional experiments. Details on this unique application of the hollow fiber method are to be found in (7,8).

B. Separation of cells by light scatter

Cytoplasmic granulation constitutes a dominant feature of pituitary cells (Fig. 4). We reasoned that this feature might be used to advantage when scattering of laser light was used as the probe. This idea has been tested using an EPICS V cell sorter equipped with an argon ion laser tuned to the 488 nm line at 150 mW. We have been able to show that the back scatter signal produced from living cells (a complex function of diffractive, reflective and refractive components) was indeed related to the degree of cytoplasmic granulation. Shown in the top panel of Fig. 5 are the back scatter (PLS-X axis) and forward angle light scatter (FALS-Y axis) signals produced by the total mixture of living pituitary cell types viewed from different projections. Also shown in Fig. 5 are the light scatter ridges after
separation using the cell sort mode of the flow cytometer. Staining analysis revealed that a) 82% of the cells in region C were GH cells, b) 65-75% of the cells in region B were PRL cells and c) 80% of the cells in region A were either follicular cells, macrophages, or endothelial cells (9,10).

The flow cytometer has proven to be an extremely useful tool not only for the separation of different live pituitary cell types (as demonstrated by Fig. 5), but also in an analytical mode where the percentage distributions of fixed and stained pituitary cell types can be objectively determined with considerable statistical accuracy in a very few minutes (11).

We have not as yet had the opportunity to attempt to sort type I and type II somatotrophs using flow cytometry. However, the instrument is proving to be extremely useful for analyzing cells separated by other methods (see later).

C. Cell separation by electrophoresis.

Our early attempts to electrophoretically separate pituitary cells employed the density gradient electrophoresis method of Boltz and Todd (12). This method required insertion of cells at the bottom of an isotonic density gradient which consists of a 5.4-6.5% sucrose gradient (bottom to top) and 2.0-8.0% Ficoll gradient (top to bottom). Electrophoresis was in the upward direction. We obtained evidence that type II somatotrophs might have a different electrophoretic mobility from cells in the type I region (13). Cells in the somatotroph enriched fraction had lower electrophoretic mobilities than those cells recovered from the band I area. However, additional analytical procedures, viz microscopic electrophoresis using a Zeiss 'cytopherometer' and automated light scattering electrophoresis using a Pen Kem 3000 Automated Electrokinetic Analyzer were also tried. Neither of these two instruments was able to distinguish significant differences in electrophoretic mobilities between band I and band II cells. Pretreatment of the cells with secretory
agents also had no significant effects on this result (see 13 for additional details.)

In 1981 the issue of whether GH cells could be electrophoretically separated from other types of hormone producing cells was reinvestigated when the continuous flow electrophoretic separator, (CFES) designed and built by the McDonnell-Douglas Astronautics Corp., became available for our use. The interested reader is referred to the chapter by Todd et. al. in this volume for information regarding the description and operation of this device.

Early trials in 1981-82 involved pituitary cell separations utilizing a low conductivity triethanolamine-potassium acetate buffer, pH 7.25, 300 mOsm/liter in the CFES device at a flow rate of 20 ml/min. The results of these early trials were encouraging in that they showed that GH cells were among the most mobile and that GH cells after CFES produced hormone and released it into the culture medium. These results were published in (13) and are not reviewed here. A second, more extensive experimental series was recently completed and published (14). Analyses of cells in the different fractions included a) counting of GH and PRL containing cells to determine their percentage distributions b) measurement of intracellular hormone by immunoassay and c) cell culture to establish the secretory capability of cells after CFE. The results of four experiments, each utilizing glands from ~50 rats, are given in Fig. 6. With no current applied cells emerged from the chamber within two outlets (1.6 mm) of the inlet port. With current applied, all pituitary cells showed anodal migration, a majority being spread over 40-50 outlet tubes (Fig. 6 panels A,F,K,N). Intracellular GH levels were 4-8 times higher in more mobile fractions (Fig. 6, panels B,G,O). Further, 60-80% of the more mobile cells were identified as containing GH by immunocytochemistry (Fig. 6, panels, C,H and L). In one experiment, intracellular LH, FSH and TSH
were also measured and found to be localized in anodal fractions (Fig. 6 panels P, Q and R). Finally, in experiments 3 and 4, GH and PRL levels in culture medium after electrophoresis were measured by immunoassay. In general, hormone output reflected GH/PRL enrichments as estimated by RIA or immunocytochemistry; a result which attests to the fidelity of separation estimates as well as inferring cell viability after CFE. The issue of the biological vs. immunological activity of the GH released from cells separated by CFE was also studied in Exp. #4 of Fig. 6. The data, summarized in Fig. 7, establish that somatotrophs in the more anodal fractions release GH which is more bioactive than corresponding somatotrophs in the less mobile areas. On the basis of these assay results it is tempting to speculate that type II somatotrophs are more electrophoretically mobile than type I GH cells in the McDonnell Douglas device.

As discussed previously (section B) the potential of the flow cytometer in analyzing cells after CFES has only recently begun to be investigated. For illustrative purposes, the results of a preliminary experiment are offered in Figs. 8 and 9. The mobility profile of the total cell population is shown in Fig. 8 (top). Counting the stained cells in this experiment by flow cytometry showed that GH cells tended to be found in two regions [fractions 11-15 and 16-20; Fig. 8 (middle)] while PRL cells were also found in two regions, but they were of lower electrophoretic mobility [fractions 6-11 and 12-14; Fig. 8 (bottom)]. Results of two parameter analyses of GH-stained cells in fraction 5 vs. fraction 19 are given in Fig. 9. In this figure the log of the green fluorescence staining (LPGFL), which can be attributed specifically to GH cells, is shown on the X axis whereas the forward angle light scatter, attributed specifically to cell size, is shown on the Y axis. All of the "events" shown in Fig. 9 (29,999 for Fr. #5 and 14,338 for Fr. 19) can be
unequivocally attributed to cell signals since they were "gated" on red fluorescence signals generated using propidium iodine. The predominant difference between GH cells in Frs. 5 vs. 19 is that a majority of GH cells in Fr. 19 are large and well defined (area labeled by ▽) whereas small, less intensely stained GH cells are common to both fractions (identified by *).

What bearing and relationships do the pituitary cell separations by CFES have on the issue of microgravity-based research? In light of the different densities of the various hormone-producing cell types, we were concerned that this parameter might contribute to the apparent electrophoretic separations actually achieved (especially those on the CFES device). However, the results of our microgravity experiment on board STS-8 in September 1983, (identified as Exp. #2 in Fig. 6), establish the general principle that we have seen consistently, viz—that a majority of the GH cells are more electrophoretically mobile than the PRL-containing cells. Therefore we feel confident in concluding that the net charge on the surface of most somatotrophs is significantly different from that of most PRL cells. This non-trivial result will be confirmed, optimized and considerably extended in a future microgravity based separation experiment (see later).

II. Purification of human growth hormone (hGH).

In a well known textbook on Endocrinology one finds the following statements concerning GH in the human pituitary gland after death:

"Fortunately, the human pituitary is particularly rich in GH, and the somatotropic granule resists autolytic dissolution after death. Despite the fact that radio-immunoassays suggest a much higher content, the yield of somatotropin with present extraction methods is between 8 and 16% of the dry weight of human pituitaries, equivalent
to 4-8 mg of hormone/gland. No significant changes in GH content with age are evident."

A number of years ago the National Pituitary Agency was established for the purposes of purifying hGH from post mortem glands and providing this material for clinical medicine. Over the years, many children of short stature benefited significantly from injections of the purified hormone. In fact, the National Pituitary Agency processed about 50,000 pituitaries a year, supplying the hGH from them to about 5000 children. Supply did not keep pace with demand. Quite recently this situation changed dramatically when the program responsible for purification of hGH was shut down when the deaths of three patients receiving hGH were attributed to a slow virus contaminating the hormone preparations (15). In the Fall of 1985 the Food and Drug Administration gave its approval for use of a genetically engineered version of hGH produced by Genentech. Patients are currently receiving this hGH at a cost of $10,000/year for three injections/week.

In section I of this report we presented evidence that there were at least two forms of GH secreted from subpopulations of rat pituitary somatotrophs. One had a significantly higher bioactivity/immunoactivity ratio than the other. Our current research efforts with human pituitary GH are based on a similar idea, viz that a discrete form of hGH with high biopotency is contained within the human pituitary. Our goal is to purify this material. The different approaches which are being used are now briefly summarized.

**Approach #1.** Continuous Flow Electrophoresis (CFE) of human post mortem pituitary granules.

Shown in Fig. 10 is an electron micrograph of the appearance of human pituitary tissue eight hrs after death. The dominant feature of this tissue, as is evident from the figure, is that it is laden with hormone-containing
granules. We reasoned that CFE of suspensions containing such particles might be a useful approach to the isolation of hGH with high biopotency. The experiment involved a) preparation of homogenate of 11 hr post mortem tissue in isotonic sucrose, b) preparation of a crude granule fraction by centrifugation at 20,000 xg, c) washing of the pellet in CFES buffer, d) CFE separation (ET150), e) collection of material from CFE into 200 individual tubes containing alkali (to disrupt granules), f) radioimmunoassay of the solubilized material in each of the 200 tubes and finally g) pooling of tubes for assay of bioactive GH via the tibial line assay. Shown in Fig. 11 is the profile of immunoreactive hGH after continuous flow electrophoresis. Although each of 200 tubes were analyzed, only #'s 90-140 are shown since the others contained no detectable activity. Clearly, there is a sharp, symmetrical peak encompassed by tubes 100-109 which contain the bulk of the immunoactive hormone. The position where most of the bioactive hGH was recovered was clearly somewhat different (Fig. 12). While 75% of the bioactive hormone was recovered in tubes 88-113, the hormone in combined fraction #4 (tubes 114-200) was obviously enriched in bioactivity.

**Approach #2.** Fractionation of hGH contained in alkaline extracts of human post mortem pituitary by high performance liquid chromatography (HPLC).

On the basis of earlier findings by Ellis and Grindeland which suggested that bioactive rat GH might be of considerably higher apparent molecular weight than the well characterized monomeric 22 kD form, some experiments have been done using HPLC on alkaline extracts of human post mortem pituitary tissue. The results of one such experiment are given in Fig. 13. Shown in the top portion of this figure is the OD 214 nm of the general protein profile after HPLC. Approximate molecular weights of material eluting in the pooled fraction areas indicated are: fr. 1, void volume; fr. 2 >117kD; fr. 3, 40-117
kD; and fr. 4 <40kD. Shown in the lower portion of Fig. 13 are data listing the contents of protein, immunoassayable and bioassayable hormone in these fractions. In this experimental series, bioassayable GH was measured by the 3T3 cell adipogenic assay of Nixon and Green (16). The data confirm the earlier observations of Ellis and Grindeland in that there is a high molecular weight form of the hormone which is enriched in bioactivity.

Approach #3. Fractionation of hGH contained in alkaline extracts of human post mortem pituitary by HPLC and CFES.

Since CFES offered promise in the separation of a GH form in secretory granules with high B/I activity (Fig. 12) we reasoned that a combination of the HPLC and CFES techniques might prove interesting. Accordingly, an extract of post mortem pituitary gland was fractionated by HPLC. As before (Fig. 13) a high molecular weight fraction was obtained which had 23 μg of bioactive hormone and 2.8 μg of immunoactive hormone. This material was fractionated by CFES into 200 individual tubes and pooled on the basis of 15 tubes/assay fraction. Results concerning the distribution of biologically active hormone (3T3 assay) indicated the surprising result that ~50% of the bioactive hormone showed cathodal migration (Fig. 14). Although not shown on this figure, only 20% of the total immunoactive hormone was associated with material in combined fractions 1-5. A major peak of immunoactive hGH was associated with combined fraction 8. These results again show the good potential of the CFES device in the fractionation of hGH with high bioactivity.

Approach #4. Separation of bioactive human growth hormone by affinity chromatography.

The results of each of the three previous approaches offer evidence to support the hypothesis that there is a form of the hGH molecule, high in biological potency, which can be isolated from a majority of the immunoactive
form. If true, a more direct approach for the isolation of bioactive hGH would be affinity chromatography. In this procedure, the hGH antibody is covalently bound to a column support. On passing an unknown sample containing immunoreactive hormone through the column, the immunoactive form binds whereas the bioactive form passes through. Recovery of the immunoactive form from the column is accomplished by running a high salt solution through to displace bound hormone. Only preliminary results have been obtained thus far using this approach. They are shown in Fig. 15 and Fig. 16. When the hormone standard was run through the affinity column, 99% of the recovered immunoactive hGH bound to the column. As a result, the B/I activity ratio of the pass through material was 9.3 (Fig. 15). When a fresh alkaline extract of human pituitary gland tissue was run through this affinity column, 100% of the immunoactive hormone bound to the column while a small, but nonetheless detectable, amount of bioactive hormone passed through (Fig. 15). It is important to indicate that this material was devoid of immunological activity. [Why more bioactive hGH is recovered than was applied is unknown. However, it is a highly repeatable observation and many relate to removal of a natural inhibitor of this bioactive form on purification].

The efficacy of the affinity chromatographic approach to the isolation of hGH is also shown by the Western blots in Fig. 16. All of the bands seen in this figure are reactive against hGH antiserum and are, by definition hGH. Identification of materials in lanes 1-6 are as follows: Lane 1, unfractionated hormone standard; lane 2, hGH in hormone standard bound to affinity column; lane 3, hGH in hormone standard which passed through column; lane 4, hGH in fresh human pituitary extract; lane 5, hGH in human pituitary extract bound to column; lane 6, hGH in human pituitary extract which passed through the affinity column. We interpret the molecular weight estimations of
the stained GH molecules to reflect monomeric hGH (22kD); dimeric GH (~40kD) and trimeric aggregates (~60kD). Clearly, the efficacy of the column in removing these immunoactive forms of hGH is documented by the data in Fig. 15 and Fig. 16.

III. Experiments previously done in microgravity.

Over the period of 1983–1985 our group has done three GH-related experiments on the Shuttle. A brief summary of the findings from each of these three experiments, together with their significance to the "GH system" as specifically related to microgravity, are presented in this section.

Experiment #1. The separation of rat pituitary GH cells from other hormone containing cell types was accomplished by CFES on the flight of STS-8. The data (Fig. 6, exp. 2) showed that GH cells have a surface charge which is different from prolactin cells, thereby indicating that differences in cell density could not explain the separations actually achieved on Earth.

While the microgravity experiment was generally successful, not all of its original objectives were met since cell recoveries from the CFES device were very low. This situation forced us to pool cells in order to have sufficient numbers to do those analyses shown in Fig. 6. Thus, we were unable to culture these separated cells for the purposes of determining whether high bioproducts were actually separated from the low bioproducers in microgravity. Considering the results of our second microgravity experiment (see below) there is some question as to how well the high bioproducts might actually function in space. The need to repeat the pituitary cell separation experiment in microgravity is critical to find out if the separated cells do retain their ability to produce bioactive GH.
Experiment #2. A preliminary pituitary cell culture experiment was also done on the flight of STS-8. This experiment involved storage of dispersed pituitary cells in sealed tubes in middeck lockers maintained at 37°C. On recovery, the cells were washed and cultured for six days to evaluate the secretory capacity of prolactin (PRL) and GH producing cells. As shown in Table 2, the secretory activity of GH cells was severely compromised by exposure to microgravity, while that of PRL cells was not. Considered with the experiences of the Cogoli group with regard to the failure of microgravity exposed lymphocytes to respond to Con A (17) and alteration of varied cell functions recorded on the German D-1 mission in late 1985, the possibility that the lack of gravity has direct effects at the cellular level appears worthy of further study. In specific regard to GH, the issue assumes added importance in terms of the documented effects of this hormone on function of muscle and bone, two tissues known to be significantly affected by flight. Another pituitary cell culture experiment has been approved for flight and currently awaits remanifesting.

Experiment #3. Pituitary GH cells contained in glands of rats flown on SL-3 in April, 1985 were also studied by our group and shown to be defective in GH secretion. The results of this study (18) showed that release of both bioactive and immunoactive GH from isolated cells after flight was reduced by about 50% relative to appropriate ground based controls. On the other hand, PRL secretion was unaffected. In this regard, the SL-3 results and STS-8 results regarding a defect in GH release are internally consistent. Additional evidence suggested that a high molecular weight form of the GH molecule, enriched in bioactivity, was not released from the flight cells.
IV. Preparation for a future flight experiment: Aims, protocols, rationale, ground based research, potential problems.

A. The experimental protocol for aim #1 of our approved ground based research and flight experiment is given below. In essence this experiment involves the separation of different rat pituitary cell types by CFE.

AIM #1 Experimental Protocol

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rat pituitary cells

\[\downarrow\]

continuous flow electrophoresis

\[\downarrow\]

fractions (10-25)

[ cell culture to assay B/I activity of secreted hormone in serum and serum-free medium ]

[ immunocytochemistry by flow cytometry to determine purity of cell type in fraction ]

[ forms of intracellular and secreted GH ]
  a) HPLC-reverse phase
  b) HPLC-sizing
  c) Western blot

[ hollow fiber implants into hypophysectomized rats ]
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With this approach we should be able to obtain answers to such questions as:

1. What is the degree of cell purification achieved by CFE under conditions where extensive cell pooling is not required (as it was on STS-8)?

2. What are the B/I activities of GH released from the electrophoretically separated cells?
3. What are the molecular forms of the secreted GH?

4. Which electrophoretically purified population of GH cells is capable of reinitiating total body growth, increases in muscle mass, and increases in organ weights?

5. What effect does microgravity have on the answers to questions 1-4?

If we are correct in our suspicion that GH cells in microgravity experience a lesion in hormone secretion, a successful outcome to this experiment will not only helped to define the cellular and molecular basis of this lesion, but will also identify the specific GH cell subpopulation (in terms of its electrophoretic mobility) that is affected by microgravity.

GENERAL CONSIDERATION AND POTENTIAL PROBLEMS TO BE ADDRESSED IN THIS RESEARCH

It is obvious that the experimental conditions found to yield optimal results on Earth should be the ones actually used in the microgravity experiment. We believe that the cell holding conditions prior to electrophoresis should be made more compatible with the physiology of the actual cell system; (e.g. under ambient temperatures in protein containing buffers). Furthermore, the issue of the use of a CFES buffer system more compatible with excellent cell viability needs to be addressed. The actual CFES run of the cells should ideally be performed early on in the flight. The separated cells should be held in culture media so that the issue of GH secretion from cells in microgravity can be investigated. Finally, it is essential that the rats be killed at KSC in order to prepare cells under conditions where the possibility of picking up contamination during cell preparation is virtually eliminated. This procedure is done routinely in our laboratory with virtually 100% success.
B. The experimental protocol for aim #2 of our approved ground based research and flight experiment is given below. In essence, this experiment involves the separation, by CFES, of variant forms of the human growth hormone molecule contained in either secretory granules/or alkaline extracts of human post mortem pituitary tissue.

Aim #2 Experimental Protocol

Affinity Purified Human Pituitary Extract/Granule Fraction

Continuous Flow Electrophoresis

Fractions (200)

Protein Analysis  B/I of Separated GH  HPLC, Blotting

With this approach we should be able to answer the following:

1. Can the CFES device be utilized to purify a hGH form which is high in biological activity, but low in immunoactivity?

2. What are the molecular forms of the GH contained in these fractions?

3. Can the amount of hGH processed through CFES be significantly increased in microgravity?

Based on the experiences of McDonnell Douglas researchers with the CFES device in microgravity, we anticipate that "throughput" will be significantly
increased in microgravity. The electrophoretic separation of a biologically potent form of hGH would be of considerable interest to the pharmaceutical industry as well as to the medical community. Obviously, any findings from this experiment could serve as the basis for future work by the such groups.

GENERAL CONSIDERATIONS AND POTENTIAL PROBLEMS TO BE ADDRESSED IN THIS RESEARCH.

Since in aim #2 we are dealing with extract/subcellular particulate material, the sample for the actual microgravity experiment can be prepared in the PI's laboratory well ahead of the launch date and be stored in a frozen state until the time of electrophoresis. After the electrophoresis run, the 200 samples can be stored at 4°C until return to Earth.
Fig. 1. Immunological and biological activities of growth hormone released from mixed, type I and type II somatotrophs over a four day culture period. Immunoassays were performed by RIA; bioassays by the tibial line assay in hypophysectomized rats. Culture medium was MEM + 5% calf serum and antibiotics.
Fig. 2. (Top) Photomicrograph of 10 mm long XM-50 hollow fiber and in cross section. (Middle) Scanning electron micrograph showing lumen and fiber wall. (Bottom) Scanning micrograph of pituitary cells in hollow fiber.
Fig. 3. Responses of hypophysectomized rats receiving hollow fibers containing nothing (empty, E); mixed (M); type I (I) or type II (II) somatotrophs 12 days post implantation.
Fig. 4. Electron micrograph of freshly dispersed rat pituitary cells. Most of these cells are GH producers. Note the large numbers of cytoplasmic secretory granules which are known to contain GH.
Fig. 5. Light scatter profiles of live pituitary cells before (A-C) and after (D-L) sorting. Cells from 46-day-old male rats were dissociated in the presence of dopamine (10⁻⁵ M) and analyzed for PLS (X-axis) and FALS (Y-axis). Note correspondence of the light scatter profiles of cells sorted from regions A (D-F, 2 X 10⁶ cells), B (G-I, 5 X 10⁴ cells), and C (J-L, 4.3 X 10⁴ cells) with the ridges marked a, b, c on the profiles generated from the unfractionated cells (A-C, 7 X 10⁵ cells).
Fig. 6. Separation of rat pituitary cells by CFES. Mobility profiles of cells are shown in panels A, F, K and N. Intracellular GH and PRL contents of cells obtained from pooled areas shown were determined by immunoassay. Percentages of GH and PRL containing cell types in these fractions were determined by immunohistochemistry. Exp. #2 was done in microgravity. All runs were at ET 150 under conditions described in the text.
Fig. 7. GH released from GH cells separated by CFE. Cells in pooled electrophoresis fractions 5+6 and 10+11 (see Fig. 6, Exp. 4, panel 0) were cultured for six days and the GH released into the medium was assayed by RIA and tibial line bioassay. The intracellular contents of GH in cells in these pooled fractions prior to culture are shown in the left panel.
Fig. 8. Distributions of GH and PRL containing cells in fractions after CFES. These distributions were determined from counts of 30,000 cells/fraction, after staining with hormone specific antibody, by flow cytometry.
Fig. 9. Two parameter flow cytometric analysis of GH cells contained in Fr. 5 (top) and Fr. 19 (bottom) after the CFES trial shown in Fig. 8. The intensity of the green fluorescence (X axis) relates to the presence of GH cells, while the forward angle light scatter (FALS, Y axis) relates to the cell size. Numbers of cells counted are shown in upper right corner. The intensely stained GH cells in Fr. 19 (identified by ▼) are not present in fr. 5. However, some less intensely stained GH cells are seen in both fractions (*).
Fig. 10. Electron micrograph of a human post mortem pituitary gland 8 hrs. after death. Although general cell autolysis is evident, the GH-containing granules are intact.
Fig. 11. Distribution profile of immunoactive human GH contained in CFE fractions after electrophoresis of a crude pellet (20,000 X g) containing granules. Although not shown on figure, tubes 1-89 and 141-200 were assayed and found not to contain GH.
Fig. 12. Biological activities of hGH contained in granule fractions separated by CFES. The experiment depicted in Fig. 11 resulted in 200 tubes which were pooled into four areas shown in the insert. Hormone in these pooled fractions was measured by immunoassay and tibial line bioassay.
Fig. 13. Fractionation of an alkaline extract of a human pituitary gland by HPLC and immunological/biological activities of the hGH contained in pooled fractions eluting from the SW300 sizing column. The OD$_{214}$ profile (at a setting of 1.0 full scale deflection) is shown in the upper panel and the hormone activities of the pooled areas are shown in the table (bottom). Biological activity of the hGH was determined by 3T3 bioassay.
Fig. 14. CFES of human pituitary extract. Fr. #2 from an HPLC run (see Fig. 13) was applied to CFES and the resulting 200 tubes were pooled (15/fraction) prior to assay by 3T3 cell bioassay. Although not shown in the figure, only 20% of the total recovered immunoreactive hGH was associated with material in frs. 1-5.
Fig. 15. Biological and immunological activities of hGH standard and a human pituitary extract before and after affinity chromatography. A highly potent hGH antiserum was covalently coupled to Pierce Reactigel (see text). Material bound to the column was removed by elution with 4M KSCN.
Fig. 16. Western blot SDS-PAGE profiles of hGH contained in hormone standard or in human pituitary extract before and after affinity chromatography. Lanes 1 and 4 show hGH in standard or fresh extract respectively. Lanes 2 and 5 show the appearance of hGH forms after elution from the column with KSCN. Note that virtually no hGH immunoactivity is present in the passthrough of either the hGH standard (lane 3) or pituitary extract (lane 6). Molecular weight markers show mobilities of known standards. See figure 15 for assay data.
TABLE 1
COMPARISONS OF TWO TYPES OF SOMATOTROPHS

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<tr>
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<th>Type I</th>
<th>Type II</th>
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<tr>
<td>Density (g/cm³)</td>
<td>1.050 - 1.068</td>
<td>1.071 - 1.086</td>
</tr>
<tr>
<td>Secretory Granules</td>
<td>Few</td>
<td>Many</td>
</tr>
<tr>
<td>Appearance (EM)</td>
<td>Abundant Regions of RER and Golgi Apparatus</td>
<td>Few Regions of RER and Golgi Apparatus</td>
</tr>
<tr>
<td>Db-cAMP (3 x 10⁻³ M)</td>
<td>Stimulated Secretion</td>
<td>Stimulated Secretion</td>
</tr>
<tr>
<td>Somatostatin (10⁻⁷ M)</td>
<td>Inhibited Secretion</td>
<td>Inhibited Secretion</td>
</tr>
<tr>
<td>Thyroxine (10⁻⁶ M)</td>
<td>No Effect</td>
<td>Stimulated Secretion</td>
</tr>
<tr>
<td>Hydrocortisone (10⁻⁶ M)</td>
<td>Increased Cell GH</td>
<td>No Effect</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Hormone Released</th>
<th>Hormone Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GH</td>
<td>PRL</td>
</tr>
<tr>
<td>Earth</td>
<td>16.8</td>
<td>3.3</td>
</tr>
<tr>
<td>Space</td>
<td>0.8</td>
<td>5.0</td>
</tr>
</tbody>
</table>

*Cells flown for 7 days on STS-8 in September 1983.*

+6 day culture; 1x10^6 cells in 5 ml αMEM + 5% calf serum.

++Hormone produced = hormone released - intracellular hormone seeded
REFERENCES


<table>
<thead>
<tr>
<th>Pooled HPLC FRX</th>
<th>Protein (μg)</th>
<th>EIA (μg)</th>
<th>3T3 (μg)</th>
<th>B/I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting Material</td>
<td>1447</td>
<td>111±3</td>
<td>48</td>
<td>0.43</td>
</tr>
<tr>
<td>1</td>
<td>58</td>
<td>6.7</td>
<td>3.1</td>
<td>0.46</td>
</tr>
<tr>
<td>2</td>
<td>54</td>
<td>6.5</td>
<td>45.1</td>
<td>6.94</td>
</tr>
<tr>
<td>3</td>
<td>725</td>
<td>245.7</td>
<td>128.1</td>
<td>0.52</td>
</tr>
<tr>
<td>4</td>
<td>92</td>
<td>11.1</td>
<td>0.95</td>
<td>0.09</td>
</tr>
</tbody>
</table>

**TABLE 6**

Fractionation of hGH by affinity chromatography

<table>
<thead>
<tr>
<th>Sample</th>
<th>Column+ Fraction</th>
<th>Bioactive hGH (μg)+</th>
<th>Immuno-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>(μg)</td>
<td>B/I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purified hGH standard</td>
<td></td>
<td>a) Unfractionated starting material</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b) Pass through</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c) Bound</td>
<td>4.8</td>
</tr>
<tr>
<td>Crude alkaline extract of human pituitary gland</td>
<td></td>
<td>a) Unfractionated starting material</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b) Pass through</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c) Bound</td>
<td>8.0</td>
</tr>
</tbody>
</table>

An IgG fraction of rabbit antiserum raised against hGH was coupled to Pierce activated imidazolyl-carbamaite (Fractogel). The starting material was prepared in 0.01 M PBS and the column run in this buffer. Bound material was eluted from the column with 4M KSCN.
TABLE 5

GH activity in crude granule fractions of human post mortem pituitary tissue after continuous flow electrophoresis+

<table>
<thead>
<tr>
<th>Combined electrophoresis fraction</th>
<th>Tube number</th>
<th>GH/fraction (ug)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Immunoactive</td>
<td>Bioactive</td>
<td>B/I</td>
</tr>
<tr>
<td>1</td>
<td>0-87</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>88-103</td>
<td>9.1</td>
<td>21.4</td>
<td>2.4</td>
</tr>
<tr>
<td>3</td>
<td>104-113</td>
<td>11.7</td>
<td>26.4</td>
<td>2.3</td>
</tr>
<tr>
<td>4</td>
<td>114-200</td>
<td>0.2</td>
<td>13.5</td>
<td>62.8</td>
</tr>
</tbody>
</table>

+1 hr post mortem pituitary gland; See Fig. 8 for distribution of immunoactive GH in individual fractions from the DFE device.

Table 4

Immunological and biological activities of GH released from cells into culture medium after CFE

<table>
<thead>
<tr>
<th>Intracellular GH (immunoassay)</th>
<th>Released GH µg/750,000 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractions+</td>
<td>µg/750,000 cells</td>
</tr>
<tr>
<td>5+6</td>
<td>1.99</td>
</tr>
<tr>
<td>10+11</td>
<td>2.03</td>
</tr>
</tbody>
</table>

+See Fig. 4, exp. #4 for positions of cells migrating to these areas.
++5x10⁴ cells/fraction cultured in 200 µl aMEM containing calf serum and antibiotics in 96 well plates (5 wells/fraction) at 37°C under 95% humidified air:5% CO₂ for eight days. GH in these media were then tested by RIA or by tibial line bioassay.
In summary, we have used the cell separation techniques of velocity sedimentation, flow cytometry and continuous flow electrophoresis to obtain enriched populations of GH cells. Since our ultimate goal is to isolate a GH cell subpopulation which releases GH molecules which are very high in biological activity (but relatively poor in immunological activity), it will be important to use a method which can effectively process large numbers of cells over a short time span. The techniques based on sedimentation are limited by cell-density overlaps and streaming. For example, pituitary FSH/LH cell types have identical densities to GH (type II) cells. While flow cytometry is useful in the analytical mode for objectively establishing cell purity, the numbers of cells which can be processed in the sort mode are so small as to make this approach ineffective in terms of our long term goals. We have shown that CFES can separate GH cells from other cell types on the basis of differences in surface charge. Importantly, the bioactive producers appear to be more electrophoretically mobile than the low producers. Current ground based CFES efforts are hampered by cell clumping in low ionic strength buffers and poor cell recoveries from the CFES device. It seems likely that future research will partially alleviate some of these problems. The already proven increased throughput of the CFES device in microgravity should provide the means to achieve our ultimate goal, viz. the large scale production of bioproducing cells so that isolation and characterization of this bioactive GH form can be realized.