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The Effect of Micro-Gravity on in vitro Calcification
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A. The culture system

Chick limb-bud mesenchymal cells differentiate into chondrocyte when plated in high density (micro-mass) culture under the appropriate conditions. In the presence of m inorganic phosphate, or comparable concentrations of an inorganic phosphate source (eg. 3 mM ßglycerophopshate) the extracellular matrix around the chondrocyte nodules mineralize. The mineral formed resembles the mineral in chick calcified cartilage at d17 in ovo. This system, characterized in our laboratory in terms of gene expression, matrix protein production and modification, and mineral characteristics, thus provides a model for studying initial events in biomineralization.

B. The purpose of the study

Earlier observations of animals flown in microgravity had produced conflicting information about the nature of the mineral found in bone. One cause for these differences was that much of the mineral had been formed on earth, and whatever mineral formed in microgravity was not formed in a reproducible fashion. Since crystals formed in micro-gravity are generally larger and more perfect than those formed under similar conditions on earth, it was hypothesized that the initial mineral crystals formed under physiologic control in micro-gravity would also be larger.

In this experiment, a cell culture model of naturally occurring cartilage is used to simulate animal cartilage calcification. The experiment focuses on mineral deposition or calcification of cartilage. The experiments were used to compare the mineral formed in the microgravity of space with that formed on earth. Cultures at two different stages of development were fixed for analyses at five points during the flight, allowing evaluation of changes in proliferation, maturation, and mineralization in the cultures. Two additional cultures were fixed after re-entry.

Results of these experiments were anticipated to provide direct insight into how calcification in cartilage and bone may be controlled in space. This knowledge is important prior to extended human stays on the space station and may also provide a better understanding

of the events involved in normal bone development on Earth. Such understanding may eventually lead to the development of improved treatments for osteoporosis and other bone disorders.

C. Summary of Results

In the C-2 experiment (STS 66), we found that mineralization started later in the cartridges (both on the ground and in hypo-gravity) than in plastic, and that mineralization appeared to be retarded in hypo-gravity. The flight experiments also showed that the cells differentiated normally, but more slowly than the ground controls, and that the matrix produced was not different from that made on the ground. Results of these studies were presented at the Fifth International Conference on the Chemistry and Biology of Mineralized Tissues (Kohler Wisconsin, October 1995; Connective Tissue Research, 1996). The purpose of the C-5 experiment was to confirm these findings.

The C-5 experiment was flown on STS-72. Because of a computer problem, cells received no gases and no nutrition. The C-7 was flown on STS-77. One set of cartridges was accidentally dropped when loading them into the rail, thus the number of cartridges flown was reduced from 12 to 10 to allow concurrent ground control experiments to be run. Ground controls were repeated a week later, however, because there was a problem with the temperature control during the flight, the concurrent ground controls were performed at a different temperature. Despite these problems, the results of the C-2 experiment were confirmed. The cells in the flight cultures did not mature, formed few cartilage nodules, and showed no evidence of mineral deposition up to a culture age of 28 days. Ground controls showed the presence of mineral (based on chemical, spectroscopic, and histochemical analyses) by 21 days. The mineral in these cultures was analogous to that found in calcifying cartilage of young chicks.

The following pages summarize the information learned in each of the experiments.

A. Preparation for Flight

1. Loading of Cultures: To determine whether cultures could be transferred after plating, cultures with 25 μg/ml vitamin C and 2.5 mM ß-glycerophosphate were lifted from the plastic culture dish on day 5 or 7 and transferred to nitex membranes in the same dish. The cell cultures continued to develop and did not appear different from those control cultures which were not transferred. Both transferred and control cultures showed x-ray diffraction evidence of mineral by day 21. Micro-mass cultures transferred to the hollow fiber cartridge also

remained viable, and continued to accumulate Ca, albeit to a lesser extent than cultures maintained in the same dish.

2. Transporting Cartridges: To avoid contamination associated with transporting individual plastic culture dishes to KSC, it was decided to transport the cultures after they had been placed in the hollow fiber cartridges. To verify that once flushed with medium the cultures could be left out of the incubator for the duration of the trip to KSC, the cartridges once loaded were flushed with medium for 2 days, and then removed from the incubator, all ports sealed, and the cartridge left on a laboratory desk for 24 hrs. Cartridges were then reattached to the fluid circulating system, and Ca content and morphology compared at d21. No differences were detectable.

3. Adding Short-Lived Supplements: 2.5, 5, 10, 20 and 25 μg/ml vitamin C-sulfate (L-ascorbate-2-sulfate) was added to micro-mass cultures in plastic dishes on day 2, and maintained in the media without replenishing - while in controls L-ascorbate was prepared fresh and added to media (25 μg/ml) every other day. Cultures, supplemented with either 3mM Pi or 2.5 mM ßGP, were fixed for light and electron microscopy on day 16 and 21. Concentrations of Vitamin C sulfate greater than 5μg/ml were toxic to the cells, whereas the matrix in those with 2.5μg/ml did not appear different from control. The pattern of mineralization in cultures with 2.5mM ßGP was comparable to that in cultures with 3mM Pi. Since ßGP hydrolysis does not start until alkaline phosphatase is produced (day 7 in laboratory controls), this supplement was selected as optimal. The buffer capacity of the media was checked over an 18 day period, and the pH in cultures with ßGP or Pi was noted to remain constant at $7.4 \pm .2$. With cultures in the hollow fiber cartridges, we examined the need to replenish media by comparing recycled media and single-passage media. Since no significant differences were observed, it was decided to allowed media to recycle through the media bags and cartridges.

4. How to Measure Mineralization:

The rate of mineral accretion in plastic culture dishes is routinely monitored by measuring 45Ca uptake; with the presence of physiologic mineral confirmed by Fourier Transform microspectroscopy. To determine whether measurement of total Calcium could replace assays for ^{45}Ca, micro-mass cultures were maintained in cartridges with the medium developed above (containing vitamin C-2 sulfate and 2.5 mM ßGP). Half the cartridges were concurrently supplemented with ^{45}Ca. Cultures were collected on the days indicated, and the Ca content determined by flameless atomic absorption spectrophotometer, or scintillation counting. There was a linear correspondence between the two measurements.

B - Flight Experiments

The cultures flown on the shuttle accumulated significantly less calcium than ground and laboratory controls. The flight cultures also appeared to proliferate extensively, but there was little maturation, and few cartilage nodules formed. While at the time of recovery the ground controls (and laboratory controls) contained a poorly crystalline apatite based on FTIR microspectroscopy, no mineral could be detected in any flight cultures.

C. Discussion

The mesenchymal cells plated in micro-mass cultures proliferated extensively during space flight, but although they did they did form a few cartilage nodules, they did not mature into hypertrophic chondrocytes. This is in agreement with reports of other cell systems which proliferate but do not mature under conditions of hypogravity:

osteoblasts
macrophages
osteocytes
glial cells
and plant cells

The failure to differentiate into a mature chondrocyte may be related to the lack of load, or cell-cell interaction, or to our failure to provide sufficient time for maturation. One cannot distinguish between the effects of launch, flight in hypo-gravity, and re-entry. The experiments did show that cultures of this sort can be maintained during space flight, can be fixed during flight, and can be analyzed by multiple techniques. The question of why astronauts (and people on bed rest) lose bone mass - remains unanswered.