FINAL NASA REPORT

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Title: Studies of Intercellular Communication and Intracellular Metabolic Responses by Bone Cells to Simulated Weightlessness.

Projects within this Program:

(1) Analysis of bone and of muscle/tendon junctions collected from spaceflights SLS-1 and SLS-2.

The analysis of tibia and humerus is described in detail in the attached report relating to the SLS-1 flight. In SLS-1 we began to collect for the first time the muscle/Achilles tendon junction to look for degeneration due to non-weight bearing. Degeneration of this junction was noted but a method for quantitative evaluation has still not been developed.

The analysis of tibia from SLS-2 flight has been described in the report attached relating to the SLS-2 study. The tendon/muscle junctions were collected from every flight and control group, something we did not have from SLS-1, but comparisons have been difficult because of lack of quantitative information.

(2) Study of bone cell activity in vitro, including spaceflight effects on matrix formation and mineralization.

We are working closely with Drs Ruth Globus and Emily Morey-Holton, both at Ames Research Center, (Moffett Field, CA.) and have grown primary cultures of osteoblasts which were flown on STS45, 53 and 55. The report is attached. The analysis consisted of electron microscopy, scanning electron microscopy, histochemistry and biochemical analysis. In general, the spaceflight resulted in lack of mineralization of the collagenous matrix.

(3) Alterations of blood flow in cortical bone due to spaceflight.

In animal studies from Cosmos biosatellite flights 1887 and 2044 we found vascular degeneration in the cortical bone following spaceflight. We looked for, but did not find, similar changes in cortical bone vasculature from SLS-1 and SLS-2. However, it should be emphasized that the Cosmos flights were significantly longer duration than the shuttle flights.

Significance and Rationale:

These three projects are complementary and evaluate spaceflight on three different levels of interest: whole animal physiology (blood vasculature changes), tissue physiology (morphology and histochemistry of bone tissue samples), and cell biology (bone cells grown and flown in vitro). To search for mechanisms of how microgravity affects cellular functions, the study of isolated bone cells is highly desirable. However, the whole animal responds to spaceflight via a complex series of biological events involving muscle, bone, cardiovascular, hormonal, neural, and immune systems. Thus each of these three projects will help describe the effects of spaceflight and microgravity on skeletal metabolism (ie, bone formation and development) but the total project will provide insight into actual mechanisms which cause these effects.

Peer reviewed publications partially supported by NCC 2-655:

(1) "Morphological studies of bone and tendon" (1992) S.B.Doty, E.R.Morey-Holton, G.N.Durnova and A.S.Kaplansky, J.Applied Physiology 73:10s-13s.

(2) "Immunocytochemistry and morphology of the cytoskeleton in osteoblasts." (1994) S.B.Doty, In: The Biological Mechanisms of Tooth Eruption, Resorption and Replacement by Implants. Z.Davidovitch, editor. Harvard Press, pp.127-134.

(3) "Pathophysiology of immobilization osteoporosis." (1995) S.B.Doty and E.F.DiCarlo, Current Opinion in Orthopedics. 6:V:45-49.

(4) "Fibronectin regulates calvarial osteoblast differentiation." (1996) A.M.Moursi, C.H.Damsky, J.L, D.Zimmerman, S.B.Doty, S.Aota and R.K.Globus. J.Cell Science. 109:1369-1380.

Not Peer Reviewed:

(5) "Studies of intercellular communication and intracellular responses by bone cells to simulated weightlessness and space flight." S.B.Doty. NASA Technical Memorandum, October 1994. pp.219-221.

MORPHOLOGY AND HISTOCHEMISTRY OF BONE CELLS AND VASCULATURE OF BONE FROM RATS ONBOARD SLS-1.

ABSTRACT

Spaceflight affects the weight bearing skeletal tissues by reducing the rate of new bone formation. This effect on the long bones of flown rats has been quantitated but the effect at the cellular level and the mechanism(s) involved are not understood. We are applying electron microscopy, coupled with histochemistry and immunocytochemistry to determine the cellular functions most affected by spaceflight. The emphasis for study of these samples from SLS-1, a 9-day mission, is on the histochemical and structural changes of the endosteal and perivascular osteoblasts found in diaphyseal bone of femur and tibia. Work is still in progress but some findings are described: (1) An expected decrease in alkaline phosphatase activity in osteoblasts from flight animals, but an increase in enzyme activity in the stromal stem cells adjacent to the osteoblast. (2) An increase in osteoclastic TRAP activity in the trabecular bone region in response to spaceflight. (3) A large increase in procollagen containing secretory granules in osteoblasts in the recovery group, and a significant decrease in granule numbers in the flight group.

INTRODUCTION

Previous studies of the problem of bone loss during spaceflight have shown that the loss was due to a reduction in new bone formation rather than the loss of bone by resorption or degradation (Morey and Baylink, 1976; Wronski and Morey, 1983; Vico, et al,1988). This result implicates the osteoblast as the responder to gravity or non-weight bearing and therefore is the primary object of this skeletal research. In addition, the vascular supply within the bone matrix may be part of the problem related to reduced osteoblastic activity, since the blood vessels not only provide nutrients to these cells, but the vessels are contained in channels lined with osteoblasts and pre-osteoblasts (Doty, et al, in press,1992). Cellular activity of the osteoblast has been determined by cell size and numbers (Jee, et al,1985), by histochemical and alkaline phosphatase activity (Doty,1985), and by bone matrix formation rates (Wronski,et al,1987). Similar studies to these will be carried out on the osteoblast population, especially as found along the endosteal surfaces and within the vascular spaces of the compact bone from the rats flown on SLS-1.

MATERIALS AND METHODS

SLS-1 was a US shuttle flight which carried 10 group housed

(AEM) rats and 19 singly housed rats (RAHF). The flight was for 9 days, with some animals sacrificed immediately following flight (R+0) and some allowed 9 days of recovery following flight (R+ML). At R+0, 5 AEM and 10 RAHF rats were sacrificed and we collected one-half of the left tibia and the Achilles tendon from each AEM rat and one-half of right tibia, right femur and right humerus from each RAHF animal. The additional AEM and RAHF rats provided similar tissue samples at the R+ML time period.

All tissues were fixed in a mixture of 2% paraformaldehyde plus 0.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2, for 18 hours at 4 degrees C. Following fixation, tissues were stored in 0.05 M cacodylate buffer, pH 7.2 containing 0.01% sodium azide. Demineralization, when applied, was done with 10% EDTA in 0.1M Tris buffer, pH 7.2, at 4 degrees C. For histochemistry, vibrotome sections were collected or frozen sections taken following an overnight cryoprotection procedure using 20% sucrose plus 5% glycerol solution.

Routine light and electron microscopy continues to be done on these samples, with emphasis on the endosteal osteoblasts and the vascular components of the compact bones. Histochemical analysis of these same cell populations is being related to the morphological studies. Histochemistry will localize alkaline phosphatase activity of the osteoblasts and perivascular cells. The NADPase activity will be used to monitor the Golgi activity of these same cell populations. Acid phosphatase can demonstrate the lysosomal activity of all bone cells, but the tartrate resistant acid phosphatase (TRAP) will demonstrate the osteoclast population very specifically. We will also use silver staining to demonstrate collagen fibril formation and procollagen granules in the osteoblasts and osteoid.

Structure of the fibril pattern within the tendons from spaceflight animals is also being analyzed by electron and light microscopy. Earlier studies from one Cosmos flight suggested that some fibril rearrangements had occured during flight.

Mineralized samples from metaphyseal bone of the humeri and compact diaphyseal bone from femurs and tibias were embedded in JB-4 methacrylate. Sections 4-6 microns thick were cut on a diamond knife, collected, and stained for osteoid with Goldners' stain. These sections demonstrated fluoresecence from the tetracycline used for bone labelling purposes (see E.M. Holton report), so that the bone label could be visualized simultaneously with the osteoid stained by the Goldner method.

Finally, study of lipid accumulation in the bone marrow and the vascular spaces of the long bones will be carried out on frozen sections from these animals. Lipid increases have been documented on previous Cosmos flights, and the possibility of

lipid induced osteonecrosis of subperiosteal bone has been suggested in our previous spaceflight investigations (Doty, et al,1990.

RESULTS

Bone Formation and Osteoblastic Activity:

In the light microscope, alkaline phosphatase was visualized by Burstone's azo dye method or by a lead capture process.

(1) In the humerus, the azo dye technique was applied to cold (-20 C) polymerized methacrylate embedded mineralized bone. This demonstrated alkaline phosphatase activity along osteoblast-lined surfaces of new bone. The results indicated for the RAHF R+0 group that the controls showed more emzyme activity than the flight group. Other groups have not been completely studied.

(2) In the AEM R+0 group, the lead method for alkaline phosphatase in the tibial cortex indicated no significant difference in activity between control and flight animals. However, the flight animals within the RAHF R+0 group showed stronger alkaline phosphatase activity than the controls. (This unusual finding will be further discussed in the section describing Electron Microscopy findings.)

(3) Using frozen sections of aldehyde fixed demineralized tibial cortex, we compared the alkaline phosphatase (azo dye method) activity of the flight animals only, from the RAHF group. The RAHF R+ML group showed much greater enzyme activity than the RAHF R+0 group. We did not compare flight to control animals in each group because of a scarcity of tissue for each analysis.

(4) Using frozen sections of aldehyde fixed demineralized tibial cortex, the Golgi activity of the endosteal osteoblasts was determined by the NADPase reaction. When we studied the RAHF group, considering only the flight animals, the R+ML group was more reactive than the R+0 group. In the AEM R+0 group, the flight animals and controls showed similar activity in the osteoblasts. Comparisons among other groups have not yet been completed.

Osteoclastic Activity in Trabecular Bone:

Tartrate resistant acid phosphatase (TRAP) activity is a specific indicator of osteoclasts in bone. Using frozen sections from aldehyde-fixed demineralized metaphyseal bone from femurs, we stained for TRAP activity using an azo dye method. We found

that in the RAHF flight animals, the R+0 group showed more activity than the R+ML group. And in the AEM R+0 group the flight animals showed similar, if not slightly greater, activity than the control group. Other groups have not yet been done nor has any electron microscopic localization of TRAP activity been carried out for the osteoclast population. This remains to be done if enough tissue is available.

Electron Microscopic Findings:

(1) In all studies of alkaline phosphatase localization, the osteoblast external cell membrane shows strong enzyme activity (Figure 1). Therefore when we found that the flight animals from the RAHF R+0 group showed more activity than the control, a finding in conflict with our previous spaceflight studies, we examined this group specifically for alkaline phosphatase localization. It became immediately apparent that the stromal cells adjacent to the endosteal osteoblasts showed intense alkaline phosphatase activity (Figure 2). This activity was much greater in the flight group compared to the controls.

(2) The Golgi localization of NADPase activity showed a typical distribution in the forming Golgi saccules and the parallel array of Golgi cisternae (Figure 3). We have not yet had the opportunity to quantitate this activity but have collected electron micrographs from the RAHF R+ML and R+0 groups and the AEM R+0 groups.

(3) Electron microscopy has shown that procollagen containing granules, derived from the Golgi, are transported to the osteoblast cell membrane adjacent to the bone matrix. These granules normally are exocytosed and the collagenous products become incorporated into the newly forming osteoid. The present studies show an increase in the numbers of granules present in osteoblasts in the RAHF R+ML flight group (Figure 4) compared to the RAHF R+0 flight animals (Figure 5).

Mineralized Bone Samples for Light Microscopy:

(1) Mineralized bone samples were prepared from the RAHF R+ML group (flight and control), RAHF R+0 group (flight and control) and the AEM R+ML (flight and control) group. These samples were sectioned at 4-6 microns thick, stained by the Goldners stain for osteoid. The sample sections also show tetracycline fluorescence which was used to indicate the quantity of bone formed during and after spaceflight. The quantity of bone formed relative to the amount of osteoid present is now being quantitated and the results are to be compared to the relative alkaline phosphatase activity in each group.

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(2) Because the methacrylate embedding was carried out at -20 C the enzyme activity for alkaline phosphatase and TRAP are still demonstrable in these plastic sections of mineralized bone. Thus in the future we can quantitate bone cell numbers and their relative activity for each of the RAHF and AEM groups described above.

Studies to be Initiated:

The histochemical analyses were carried out first because (a) these enzyme activities decrease with time in storage, and (b) there is limited tissue for study so that not every analysis which we hoped to do will be possible to complete.

The vascular studies have only recently begun. In the light microscope some evaluation of the vasculature in cortical bone has been carried out, but the all important electron microscopic studies have not been done. Tissues from most groups have been embedded for future study.

The lipid distribution in bone and blood vessels, and osteocytic necrosis, has not been studied. Tissue samples for this work will be limited so this study will be reduced in priority.

The immunocytochemistry has to be drastically reduced because of the lack of tissue. However, we still plan to carry out some localization of the cytoskeletal elements in osteoblasts, at least for light microscopic study. We made several attempts to use an antibody to localize collagen degradation products in our bone samples but since the antibody did not cross react with rat proteins, the localization could not be done. It is becoming apparent that not all the immunocytochemistry we wish to do can be completed because of the lack of specific rat antibodies for certain proteins.

DISCUSSION

A great deal of work still remains to be done and thus these results have not been completed to the point of providing a full discussion of the SLS-1 flight experiment. However, several interesting observations have been made to date. **First**, the bone formation (alkaline phosphatase activity) and bone resorption (TRAP activity) show inverse relationships. Thus in the flight animals, we have reason to believe that after 9 days of flight, the formation rate in the cortex is reduce whereas the trabecular bone resorption is increased. **Second**, the increase in alkaline phosphatase activity of the stromal cells, as seen by electron microscopy, may be another response to short term spaceflight. Typically, the well differentiated osteoblast shows a reduction in alkaline phosphatase activity, however the stromal cells have not previously been studied. Thus an increase in activity during spaceflight may reflect a change of activity (differentiation ?)

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in these undifferentiated cells which may be precursors to bone cells. **Third**, procollagen containing granules in the osteoblast's cytoplasm reflect the "secretory" activity of the osteoblast and this function may be very sensitive to the presence or absence of gravity. The "synthesis" of the procollagen products via the Golgi apparatus may be responsive to spaceflight or non-weight bearing but is more difficult to quantify by morphological parameters. Thus the increase in these granules in the R+ML group and their reduction in the R+0 group accurately reflects a highly specific gravitational effect on the osteoblast function. For this reason, the machinery which moves and regulates the secretory granules, the cytoskeletal/microfilament system, will be a primary area for study in the remaining tissue samples.

Report on SLS-2 Study.

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Introduction.

It has been well documented that spaceflight and microgravity lead to a reduction in bone formation in the weight bearing bones (Morey, 1983) and this is reflected in a reduction in osteoblastic alkaline phosphatase activity (Doty, et al, 1990). Although alkaline phosphatase activity has historically been associated with new bone formation (Robison,1923) work by Bianco, et al (1993) has shown that the osteoblast pre-cursor or stromal cell also contains this specific enzyme. Their work showed that the two major differences between the stromal cell and the mature osteoblast was that the stromal cell was a dividing cell and was negatively stained for bone sialoprotein whereas the mature osteoblast did not divide and was positively stained for bone sialoprotein. Thus there are methods to distinguish between these two cell types even though they are both positively reactive for alkaline phosphatase activity.

Another interesting use of alkaline phosphatase localization in bone is to demonstrate an effect on spaceflight on the vascular system within bone matrix. We have shown (Doty, et al, 1990) that vascular ischemia can occur in vessels within bone due to spaceflight and this produces a reduction of alkaline phosphatase activity within the cells of the vascular channel. These changes are only seen in the vascular compartment within the bone matrix and are not seen in vascular systems of the adjacent muscle attachments or within the marrow space.

In SLS-2 we have used alkaline phosphatase histochemistry for light and electron microscopy to evaluate stromal cell, osteoblast and vascular cell response to a 14 day spaceflight and to a 14 day recovery period following flight.

Materials and Methods.

(1) Alkaline phosphatase histochemistry.

All tissues were preserved in EM fixative for both light and electron microscopy. This consisted of 2% paraformaldehyde and 0.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4 for 18-24 hours at 4C.

For light microscopy samples of distal femur, proximal tibia and proximal humerus were dehydrated in alcohols and infiltrated in the cold with JB-4 methacrylate resin. The tissues blocks were polymerized in a freezer at -20C to prevent enzyme damage due to the heat of polymerization. Sections of mineralized bone were cut

at 1-2 microns and placed onto poly-lysine coated slides to air dry. The alkaline phosphatase histochemical media uses an indolyl phosphate as substrate and tetranitro BT as capture agent (van Noorden and Jonges, 1987). The "intensity" of the reaction is similar wherever there is positive reaction, however the "distribution" of the reaction was variable and was quantitated with image analysis (see below).

For electron microscopy, the bone samples were decalcified and blocks of tissue were cut with a vibrotome into 50 micron thick sections. These sections were incubated in the media described by Mayahara, et al (1967) which uses beta-glycerophosphate as substrate and lead citrate as the capture agent. The reaction can be visualized as an electron dense deposit in electron micrographs.

(2) Tartrate resistant acid phosphatase histochemistry (TRAP).

The same JB-4 embedded blocks of bone tissue used for alkaline phosphatase activity could be stained for TRAP reaction. This enzyme is localized to osteoclasts and image analysis can be used to evaluate the numbers of osteoclasts per area of trabecular bone. Instead of counting osteoclast numbers we used the computer to provide us with the number of pixels which were related to the colored enzyme reaction, expressed as a percentage of all the pixels in the field. Standard field size outlined by a 4X objective was 2.4 mm2.

(3) Electron microscopy.

Small blocks of tissue sample from femur, tibia and humerus were processed through osmium, alcoholic dehydration, and embedded in Spurr's resin. Thin sections were collected on water containing brom-thymol-blue as indicator to maintain pH at 8.0 or greater (this prevents demineralization of the bone in the section). Sections were counterstained with lead citrate and alcoholic uranyl acetate and photographed with a Philips CM-12 electron microscope.

(4) Image analysis.

The sections of mineralized bone embedded in JB-4 resin were processed for alkaline phosphatase activity. The four major groups (R+0 and R+ML, Flight vrs Flight Control) were all incubated together for the same time and in the same media. The sections were viewed on a Nikon Ultraphot with a Sony video camera in place of the camera. The image was captured using Image I software (Universal Imaging Corp., West Chester, PA.) with constant magnification and constant light source voltage. Using a 40X lens the bone area on the monitor covered 0.02 mm2, the 10X lens covered 0.34mm2, and the 4X lens covered 2.4mm2. The bone area was imaged on the monitor, and the <u>color</u> of the alkaline phosphatase reaction product was highlighted and separated from all background color. The % of pixels which corresponded only to this specific color was expressed as a percentage of all pixels contained within the area of bone on the screen. Thus by choosing the appropriate magnification, a constant surface area of bone would be covered in

every sample and the reaction product expressed relative to that constant area. Therefore the higher the percentage of pixels expressed, the greater the distribution of alkaline phosphatase reaction product in that area of bone. Intensity of reaction product was not determined since the kinetics of this reaction is not known for tissues embedded in JB-4 plastic resin.

Results.

Enzyme Histochemistry. The alkaline phosphatase (AP) distribution along the humerus endosteum showed a slight reduction in activity at recovery (R+0), comparing the flight to the flight controls. The percentage of pixels indicating AP distribution within the field of view showed the flight group with 5.4 +/- 2.8% (n=6) and the flight control group with 7.1 +/- 0.6% (n=6). However there was a large difference between these two group at recovery + mission length (R+ML). The flight group after 14 days recovery on earth indicated AP distribution which was very similar to that at the R+0 time point whereas the R+ML flight controls had increased their percentage of area showing AP activity. The R+ML flight group (n=5) contained 5.8 +/- 2.1% pixels overlying AP reaction whereas the R+ML flight controls(n=6) had a value of 12.7 +/- 2.0%.

We did not have enough diaphyseal bone from the R+0 tibia samples to quantitate the alkaline phosphatase activity as done for the samples of humeri. However we did have diaphyseal bone from the R+ML group and quantitated the alkaline phosphatase in the same fashion as done for the humeri. This showed that the flight group (n=6) contained 7.05 +/- 1.6% of the total pixels over reaction product, whereas the flight controls (n=5) demonstrated 9.02 +/- 1.6% pixels related to enzyme activity. Thus the R+ML group continued to show a higher alkaline phosphatase activity in the controls compared to the flown animals even after 14 days of recovery.

In an effort to evaluate bone resorption, we made measurements on the samples of humerus using the TRAP reaction and quantifying the percentage of pixels which overlay this reaction. At R+0, the flight group (n=6) value was 2.3 +/- 0.9% and the flight controls (n=4) had a similar value of 2.2 +/- 0.3%. There was not much difference in the R+ML samples, as the flight group (n=6) value was 2.4 +/- 0.6% and the flight controls (n=6) value was a little lower at 1.8 +/- 0.8%.

Figures:

Figure 1 shows the comparison of the alkaline phosphatase distribution along the endosteum of the flight vrs the flight controls. The control tissue demonstrates a greater distribution of reaction product due to the increased activity associated with the stromal cells.

Figure 2. This electron micrograph demonstrates the alkaline phosphatase reactivity among the osteoblasts and stromal cells lining the endosteal surface of the tibia. The dense reaction

product is located along the external surface of the osteoblasts and the stromal cells. The stromal cells are distinguished by their lack of cytoplasmic organelles and undifferentiated morphology compared to the adjacent osteoblasts.

Morphology.

Light microscopy. Samples of femur, tibia and humerus were embedded in JB-4 methacrylate and sectioned as mineralized tissues. The samples were routinely stained with methyl green and pyronine to indicate the cytoplasmic RNA content of the osteoblasts. However the cytodifferentiation of these cells was so variable along the metaphyseal and endosteal surfaces, that we could make no conclusion about the effect of flight on the differentiation process. The osteoid (unmineralized bone matrix) was visualized by Goldners stain, but this too was quite variable and measurement of the osteoid did not produce any useful information.

Electron microscopy. Endosteal osteoblasts were studied from flight and flight control samples at R+0 and R+ML time periods. These cells were generally similarly differentiated, with abundant rough endoplasmic reticulum, large Golgi regions, heterochromatic nuclei, and secretory granules distributed within the cytoplasmic region. The only significant change between flight and flight controls was the distribution of the secretory granules and the presence of a well defined ectoplasmic region at the bone cell - matrix interface. In the controls, there were secretory granules located at the cell/matrix interface in noticeably large numbers. Also there was usually a well defined ectoplasmic region at the cell/matrix interface which we have described previously as indicative of collagen secretory activity. In the flight animals, even in the 14 day recovery group (R+ML) there were fewer secretory granules at the cell/matrix interface compared to the controls. However these secretory granules were present in the cytoplasm, especially in the region of the Golgi complex. The general reduction in ectoplasmic regions among these osteoblasts also suggested that overall collagen synthesis was reduced in these cells.

The vascular system within the diaphyseal bone was not morphologically different among the different groups, based on their electron microscopic structure. However we did not have access to the mid-diaphyseal region of the femur and tibia, which is where some vascular change has been noted previously, so we cannot comment adequately on this region of the vasculature.

The osteoclasts and osteocyte cell populations in the flight and control groups showed normal morphology.

Figures.

Figure 3. Comparative electron micrographs showing the distribution of the secretory granules in the flight and flight-control, especially along the cell/matrix interface.

Figure 4. Electron micrographs of the ectoplasmic region of an

osteoblast from the flight-control compared to a similar region from the flight endosteal osteoblast.

Discussion

The histochemical distribution and quantitation of alkaline phosphatase activity among the endosteal osteoblasts of the humerus and tibia, supports the conclusion that bone formation is inhibited by spaceflight. The finding that was unexpected was that in the 14 day recovery group (R+ML) the alkaline phosphatase activity was still lower than normal, and in fact, was not different than the activity measured in the same animals at R+0. Based on the tetracycline uptake data, mineralization rates were back to normal at 14 days post flight. Therefore,it may be possible that bone formation at the endosteum (as indicated by alkaline phosphatase activity) has not recovered but there is a return of normal mineralization (measured by tetracycline uptake) at the periosteal surface. The continued inhibition of new matrix formation is also supported by the morphological findings in the endosteal osteoblasts in which secretory granule and ectoplasmic expansion has not been found at the cell/matrix interface. We have shown (Doty and Schofield, 1990) that the collagen containing secretory granules are brought to the cell/matrix interface, possibly by the cytoskeletal system (Doty, 1994, in press) and released through the ectoplasmic region in contact with the bone matrix. Therefore, in this study, the reduced numbers of granules at the cell/matrix interface and the relative absence of ectoplasmic expansion along the bone surface, would support the alkaline phosphatase results, suggestive of a continued inhibition of new bone formation along the endosteal surfaces. It needs to be emphasized that endosteal bone formation may not mimic exactly the periosteal formation during the recovery phase.

It has been suggested that the first event in stimulation of new bone formation must be an osteoclastic resorption of bone (Parfitt,1988). If this event does not occur, and we saw no evidence for such activity in this study, then new bone formation may not occur. In short, the inhibition of new bone formation following flight may be explained by an absence of any initial bone resorption activity. Certainly under the conditions of spaceflight there was no obvious stimulus which would generate an osteoclastic response. And we never saw osteoclastic activity along the endosteal surfaces in the diaphyseal region of the long bones.

The increase in alkaline phosphatase activity was actually measured by the increase in the <u>distribution</u> of the activity among the osteoblasts and the pre-osteoblasts or stromal cells. Therefore the slow recovery from the flight as measured by reduced alkaline phosphatase activity may be explained by a reduction in osteoblast recruitment from the stem cells or a reduced number of stem cells or stromal cells. We presently have no information on the availability of these cells during the recovery phase.

It was noted that there was no obvious morphological change in the vascular component of the diaphyseal bone as we have seen in previous Cosmos flights (Doty, et al, 1990,1992). However we did not have access to the mid-diaphyseal portion of the long bones in this flight and therefore did not have the same regions to study as in the Cosmos flights.

Finally, we will continue to evaluate the muscle and tendon attachment to periosteum of the long bones, as the tendons have been shown previously to undergo morphological changes with spaceflight (Doty,et al,1992; Tidball and Quan,1992). These tissues which are normally subjected to intense mechanical strain should show significant changes during spaceflight and non-weightbearing.

STUDIES OF INTERCELLULAR COMMUNICATION AND INTRACELLULAR RESPONSES BY BONE CELLS TO SIMULATED WEIGHTLESSNESS AND SPACE FLIGHT

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Description of Research

Non-weight bearing and space flight cause a reduction in new bone formation, especially in the weight bearing portion of the skeleton. The cellular mechanisms responsible for this reduction are unknown although the bone-forming cells appear to be the cells most affected. Our present hypothesis suggests that the cytoskeleton of these cells could be the cellular element most sensitive to changes in gravity or microgravity. The cytoskeleton is responsible for cell shape, cell movement and attachment, and cell secretory activity. For cells involved in collagen synthesis, the secretory granules which contain pro-collagen are moved from the Golgi region of the cell to the cell membrane and then released to the extracellular space. This activity requires an intact cytoskeletal system. It has been shown that, in bone-forming cells, when the cytoskeleton is disrupted, collagen synthesis and secretion are reduced or inhibited. Previous studies have shown that the collagen-containing granules in these cells following space flight are reduced in number.

We are studying the bone-forming activity of isolated cells in order to simplify and manipulate bone formation and mineralization during space flight. This method involves growing isolated rat bone-forming cells in a three-dimensional culture system. These cells produce significant collagen matrix which will mineralize in culture. The mechanism of cell attachment, the cytoskeletal components, and the collagen secretion mechanisms are much easier to study and manipulate in this system compared to the whole animal studies discussed in the previous paragraph.

We are also continually developing new methods to localize and quantitate specific enzyme activities associated with bone-cell activity. We are quantitating the ability of bone cells to form new matrix and making these measurements at specific sites within bone samples. The technique involves our standard histochemical procedures coupled with a new imaging system and video enhancement of the sample.

Accomplishments

The most recent whole animal studies involved analysis of long bones from Space Life Sciences-1, a 9-day flight onboard Space Shuttle *Columbia*. The findings from these analyses indicated the following: (a) Stromal cells (pre-bone-forming cells) showed significantly more alkaline phosphatase activity in the flight animals than in their controls. This was the first time for this finding and was the reverse of what was found in the differentiated cell population. (b) Secretory granules in the bone-forming cells were significantly increased when the flown animals were given 9 days to recover from their flight. This suggested a reduction in secretory granule formation during the flight itself. (c) The vascular supply to the long bones did not show the extreme degenerative changes we found previously in the Cosmos series of flights.

The bone-forming cells grown in three-dimensional culture will form mineralized bone in the laboratory. We have recovered cells from three Shuttle flights (STS-45, STS-53, and STS-55) and found that they produce a collagenous matrix but have not yet found any mineralization.

This result may be partially explained by the difficulty of maintaining optimal culture conditions during flight. However, if the absence of mineralization, which is also what we found to occur in the whole animal studies, is a consistent finding, then we have the basis for future studies of cellular mechanisms.

By combining our histochemical methods with the video image analysis system, we are quantitating alkaline phosphatase activity (a measure of bone formation) on a site specific basis in each bone obtained from space flight or non-weight-bearing experiments. The results indicate that the total alkaline phosphatase activity in trabecular bone is greater than in compact bone; however, the individual cells in compact bone show greater activity than those same cells along trabeculae. Thus, site specificity becomes important in the understanding of how bone responds to space flight and non-weight bearing.

Figure 1. A scanning electron micrograph of a single bead (B) partially covered with bone cells. The cells have two general morphologies; a rounded plump cell (arrowhead) or flattened cells with numerous cytoplasmic projections (arrows). Magnification: 2,400X.

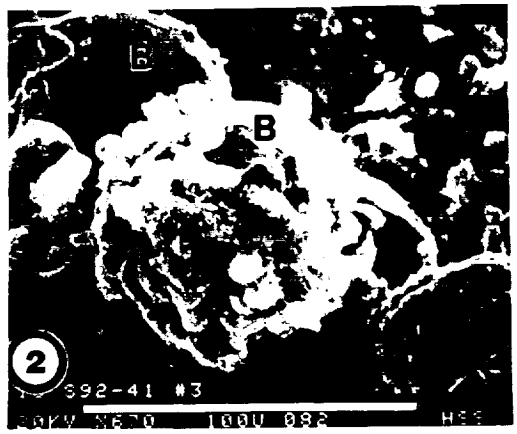

Figure 2. This scanning electron micrograph shows how the cells cover the beads (B) and how the beads and cells mixed together form a mat-like structure in the background. Magnification: 670X.

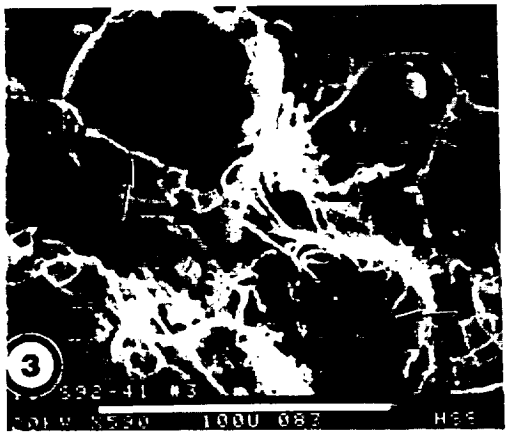

Figure 3. This scanning micrograph shows collagen strands (arrows) stretching between beads as well as attached to them. Magnification: 590X.

Significance of Accomplishments

The whole-animal studies validate our assumption that the bone-forming cells and their cytoskeletal elements should be the major focus of our continuing studies. Quantitation of the secretory granules and analysis of the cytoskeleton components will continue to be related to other data collected from biochemical and biomechanical studies.

The ability to manipulate and study in detail the cellular mechanisms involved in bone formation is greatly enhanced by the use of bone cell culture. In culture conditions, cell attachment, cell secretion, and matrix formation and mineralization can be selectively inhibited or enhanced. The cellular responses to these changing activities can then be compared to the response found in the whole-animal studies following space flight.

The quantitation of enzyme activities, especially for the alkaline phosphatase activity, is especially important for analysis and description of the mechanism of matrix mineralization. This analysis will help to separate the mineralization process from the collagen secretion process and thus improve our understanding of how space flight or non-weight bearing affects new bone formation.

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