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PROSPECTS

Utilization of Microgravity Bioreactors for Differentiation of Mammalian Skeletal Tissue

Brenda J. Klement and Brian S. Spooner
Division of Biology, Kansas State University, Manhattan, Kansas 66506-4901

Abstract Bioreactor cell and tissue culture vessels can be used to study bone development in a simulated microgravity environment. These vessels will also provide an advantageous, low maintenance culture system on space station *Freedom* Although many types of cells and tissues can potentially utilize this system, our particular interest is in developing bone tissue We have characterized an organ culture system utilizing embryonic mouse pre-metatarsal mesenchyme, documenting morphogenesis and differentiation as cartilage rods are formed, with subsequent terminal chondrocyte differentiation to hypertrophied cells Further development to form bone tissue is achieved by supplementation of the culture medium Research using pre-metatarsal tissue, combined with the bioreactor culture hardware, could give insight into the advantages and/or disadvantages of conditions experienced in microgravity. Studies such as these have the potential to enhance understanding of bone development and adult bone physiology, and may help define the processes of bone demineralization experienced in space and in pathological conditions here on earth © 1993 Wiley Liss Inc

Key words: bone, morphogenesis, cartilage rods, space

MICROGRAVITY, TISSUE CULTURE, AND BIOREACTORS

The potential utility of a microgravity environment for the advancement of biological and biomedical knowledge is largely unknown. In particular, the advantage of low gravity conditions, with decreased mechanical stress, on cell and tissue activities that include morphogenesis and differentiation are poorly understood. The paucity of knowledge in this area may be due to the limited access available for experimentation in a microgravity environment (i.e., orbital spaceflight). This has resulted in a very slow progression in advancement of scientific and biomedical research concerning the potential advantages of reduced gravity on cellular physiology. Simulated microgravity conditions may help to improve our understanding of gravitational influences on basic biological processes, with the substantial advantage of allowing studies to be done here on earth.

Rotating tissue culture vessels, developed and characterized by NASA, are designed to simulate the conditions of microgravity [Wolfe and Schwarz, 1991]. The cells and tissues are suspended in fluid by constant turning, in an essentially freefall state. This type of culture system offers many advantages including 1) the exertion of low shear forces across cell membranes, as would be encountered in a low gravity environment, 2) an automatic continuous and constant replacement of culture medium, and 3) suitability for use with both suspension and anchorage dependent cells, as well as small tissues. These characteristics may prove to be advantageous because individual cells will be allowed to grow three-dimensionally, in contrast to the two-dimensional growth of cell cultures on earth due to the limits imposed by gravity. These devices can also be used to study cell and tissue growth on space shuttle as well as on space station *Freedom.*

EFFECT OF MICROGRAVITY ON BONE

The increase in human exploration outside of earth's gravitational boundary has brought about a great deal of concern because of the deleterious effects of microgravity on the musculoskeletal system. Prolonged exposure to a microgravity environment results in bone demineralization, abnormal collagen deposition, and an alteration in the osteoblast precursor popula-

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Address reprint requests to Dr Brian S Spooner, Division of Biology, Kansas State University, Manhattan, KS 66506-4901

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tion. Preliminary evidence suggests that the bone forming cells, as opposed to the bone resorbing cells, are the population that is most affected in microgravity. The osteoclast population does not seem to be hindered, resorption of bone occurs at a rate similar to that seen in normal gravity [Vico et al., 1988].

Tetracycline labelling of rat bones before and after spaceflight show very little osteoid matrix production between the two labelling periods [Morey and Baylink, 1978], and the osteoid that is deposited is arranged in an abnormal manner [Turner et al., 1985]. Osteoblast histogenesis has been analyzed by measuring nuclear size, using the periodontal ligament of rats which have undergone spaceflight [Roberts et al., 1981; Garetto et al., 1990]. This is a well defined kinetic model for assessing proliferation and differentiation of cells associated with the osteoblast lineage. The results indicated that there was an increase in the less differentiated osteoblast lineage cells and a decrease in the cells which were more differentiated, suggesting that a block in sequential differentiation had occurred. However, a strong burst of osteogenic activity was observed in the postflight period.

Furthermore, evidence indicates that, in addition to a decrease in bone synthesis, the small amount of matrix and mineral crystal that do get deposited in growing rats experiencing a reduced gravitational environment, appear to be arranged in an abnormal pattern [Spengler et al., 1983]. The hydroxyapatite crystals themselves appear normal, but the deposition in the osteoid matrix is aberrant. Similarly, the alignment of collagen fibers is abnormal [Turner et al., 1985]. Both of these factors appear to be responsible for what is seen as an arrest line in the bones affected by microgravity. These defects may in part help explain the detrimental effect of microgravity on the torsional mechanical properties in bone synthesis. An experiment onboard *Cosmos 936* utilized a centrifuged group of flight rats. These centrifuged rats showed more normal mechanical properties of bone, indicating that distribution of osteoid matrix and mineral crystals were normal, but the proportion of newly synthesized bone matrix remained at the same level as the uncentrifuged flight group [Spengler et al., 1983]. This suggests that both quantity and quality of bone are affected during spaceflight.

The ultimate goal of microgravity research on bone is to provide solutions for alleviation of physiological problems associated with extended reduced gravity exposure, while at the same time generating answers and therapies for diseases of bone that occur at unit gravity. Elucidation of the mechanisms responsible for the altered bone physiology under microgravity conditions may be more readily attainable if the system studied is simpler than the intricate complexities of the adult, such as newly forming embryonic bone. Analysis of initial long bone formation, documentation of the stages in development, and the cellular sub-population that may be influenced by microgravity or simulated microgravity may provide clues to the mechanisms acting in adult bone demineralization.

EMBRYONIC BONE FORMATION

During embryonic bone formation, each new developmental step is sequentially added to the primitive bone anlage, to ultimately form the structure that persists throughout the organism's life. In particular, populations of mesenchyme cells differentiate into chondrocytes, which are morphogenetically organized as hyaline cartilage. These cells subsequently undergo morphogenetically patterned terminal differentiation into hypertrophied chondrocytes, which tissue specialize as calcified cartilage. Another wave of mesenchyme cells invade the calcified matrix, where they differentiate as osteoblasts and deposit a mineralized matrix or bone (Fig. 1).

We have recently conducted a series of studies expanding the sequence of developmental steps that can be achieved during continuous culture of pre-metatarsal mesenchyme tissue, documenting morphogenesis and differentiation by histological and biochemical criteria. Use of this system may provide valuable clues for understanding bone physiology, and moreover, has the potential for growth and experimentation in alternative environments such as the bioreactor system.

The culture system consists of three middle pre-metatarsals which have been excised from mouse embryos at 13 days of gestation. At this stage mesenchyme cells are arranged in a tight group, or condensation, in the approximate areas where cartilage will form. The explants are placed in our culture apparatus with chemically defined nutrient medium [Klement and Spooner, 1992]. By 3 days of culture, the mesenchyme cells have differentiated into chondrocytes and have synthesized an abundant extracellular ma-

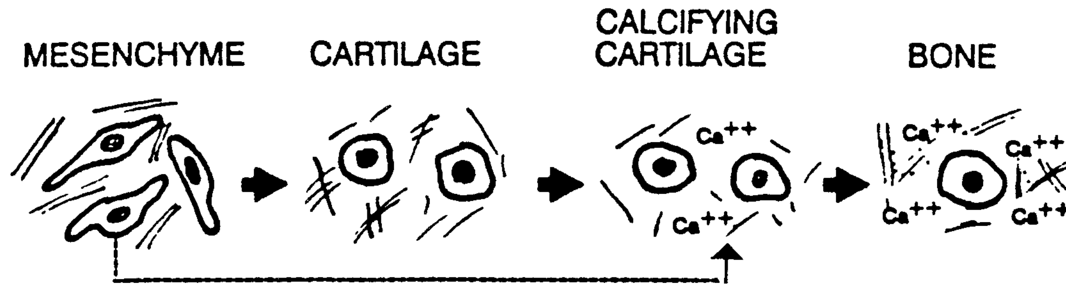

Fig. 1. A diagrammatic scheme of the cellular changes in long bone formation On the far left, mesenchyme cells are initially undifferentiated, and arranged in a tight group A portion of these cells differentiate into chondrocytes, forming cartilage tissue, and become arranged in discrete rods A sub-population of these chondrocytes undergo terminal differentiation, resulting in hypertrophied cells, surrounded by a calcifying extracellular matrix The hypertrophied cells die and are replaced by another type of mesenchymally derived cell, osteoblasts, which lay down the osteoid matrix

trix. They have formed cartilage tissue. More interestingly, the cartilage rods that form in culture have the same characteristic morphology as the in vivo pre-metatarsals [Klement and Spooner, in press]. After 10 days of culture, cells in the central region of the rods have terminally differentiated into hypertrophied cells. The matrix around these cells becomes calcified, demonstrated by alizarin red staining of the calcium deposits in the tissue. Again, a spectacular morphogenetic patterning has occurred with this differentiation step. Only the cells in the central portion of each rod have undergone terminal differentiation, while the two ends remain as hyaline cartilage [Klement and Spooner, in press]. This is the same patterning as occurs during in vivo development. This culture system thus allows morphogenetic patterning during three-dimensional development of the tissue, even at unit gravity.

A dramatic series of changes in gene expression underlie the differentiation events seen in these cultures. Since transitions in collagen isotype synthesis are molecular markers of such changes, and since type II collagen is fairly unique to cartilage, we have been assessing collagen biosynthesis in these cultures. Radiolabelled glycine can be metabolically incorporated into collagen α chains, in these cultures, which can be resolved on SDS-PAGE after the tissue has been homogenized and pepsin digested. Autoradiograms are made from the resulting gel, and the density of the α chain bands can be measured with a densitometer. Densitometry of the autoradiograms reveals relative degrees of radioactivity of α chains and, by calculation, collagen isotypes [Klement and Spooner, in press].

This approach has demonstrated the initial appearance of type II collagen (i.e., cartilage collagen) as the mesenchyme first differentiates into cartilage in these cultures, relative increases in type II collagen during continued hyaline cartilage differentiation, and a general pattern of increased synthesis of resolvable cartilage collagens (types II and XI) until terminal chondrocyte differentiation is well underway. Type I collagen is synthesized throughout the culture period, consistent with the histological documentation of mesenchymal populations around and between the rods and cellular heterogeneity of the dynamically developing rods themselves.

While collagen type II serves as a molecular marker of cartilage differentiation, we chose alkaline phosphatase activity as a marker for initiation of terminal differentiation into calcified cartilage and bone. One isoform of this enzyme is specific for mineralizing tissues and has a characteristic heat sensitivity [Bourne, 1974; Posen et al., 1965]. We have assayed enzyme activity in homogenates of cultured explants, which show a dramatic elevation of alkaline phosphatase activity coordinating with the onset of hypertrophy [Klement and Spooner, 1992]. Furthermore, the elevated activity is restricted to the central hypertrophied zone in these tissues, where calcification is taking place [Klement and Spooner, 1992]. Thus, alkaline phosphatase activity serves as a molecular marker for the second differentiation stage observed in these cultures, the change from hyaline cartilage to calcified cartilage.

This culture system provides an elegant vehicle for the study of embryonic bone formation. The explanted tissue consists of a heterogeneous population of cells which provides a more realistic model of normal, in vivo, developing tissue, while, at the same time, the explant is

totally isolated from the rest of the embryo (and potential stimulating factors) and the mother. This is a desirable condition because it provides an opportunity for manipulation of specific environmental parameters, and analysis of the effects of these alterations on differentiation and morphogenesis. In view of this potential, initial experiments using the characterized organ culture system were undertaken to determine if an additional developmental step could be achieved, such as bone formation. One proposal was to reduce the time period in culture required for terminal differentiation to occur, theoretically leaving additional time before tissue necrosis began (about 15 days) for additional developmental steps, like osteoblast differentiation, to take place. Addition of a fully defined chemical medium supplement, ITS (insulin, transferrin, selenium), to the culture medium resulted in terminal chondrocyte differentiation in 7 days instead of the usual 13–14 days we observed with unsupplemented medium. However, we still did not see bone tissue form, even though shortening the time to cartilage hypertrophy expanded the time available for such differentiation [Klement and Spooner, in press].

The next logical step was to supplement the medium with serum, a rich, but undefined, source of vitamins, growth factors, and stimulating agents. Again, supplementation results in terminal chondrocyte differentiation within 7 days. However, with additional culture time, an osteoid-like matrix is synthesized by about 12 days of culture [Klement and Spooner, in press]. This demonstrates that manipulation of the system is possible and offers a promising method for better understanding the molecular details and influences involved in bone formation.

Embryonic mouse pre-metatarsal tissue would be an excellent system for experimentation in a bioreactor vessel. The well-characterized differentiation and morphogenetic events we have described would be an excellent model for analysis of potential changes or enhancements brought about by simulated microgravity conditions in the bioreactor.

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Mechanical forces, such as the pull of muscles and weight bearing, can be simulated in a culture system by use of intermittent compressive force. Burger and co-workers [1991] used this system on cultures of embryonic mouse pre-metatarsals to analyze mineralization components. Exertion of intermittent force, near physiological range, on pre-metatarsals undergoing chondrocyte hypertrophy, accelerated the onset and increased the degree, of mineralization, compared to control cultures [Klein-Nulend et al., 1986]. Decreased mineral resorption, accompanied by decreased numbers of osteoclasts in the diaphysis, were also observed [Burger et al., 1989; Klein-Nulend et al., 1990], as were changes in sulfate metabolism and proteoglycan content [Bagi and Burger, 1989]. These results suggest that biomechanical forces play a role in the mineralization process, but the actual mechanisms are poorly understood.

It would be interesting to test this intermittent compressed force culture system in a microgravity environment to see if similar accelerated mineralization and other changes in cellular activities are obtained, or if a normal developmental pattern, like that on earth, is seen. Such studies may allow determination of the importance of physiological force in development, and suggests approaches to potential biomedical applications. The bioreactor culture system should also provide information on the importance and mechanisms of external forces, such as gravity or reduced mechanical shear, on the development of bone. It would almost provide the opposite extreme from an intermittent compressed force system, generating environmental similarities to those found in a microgravity environment. It could be extremely informative, and potentially have significant biomedical value, to assess alternating compressive forces with simulated microgravity in bioreactor studies with developing bone.

Bruder and Caplan [1989] have noted that different populations of osteogenic precursors can be delineated by specific differentiation markers on the surface of these cells. Antibodies to these markers have been used to locate each sequential differentiated cell type throughout the periosteal and bone tissue [Bruder and Caplan, 1990]. Use of antibodies would provide a method for osteoblast lineage analysis, in addition to nuclear size as a marker, which was demonstrated in the periodontal ligament. Combining use of these antibodies with an embryonic bone culture system, in a bioreactor vessel would enable detection of any anomalous differentiation in the osteoblast precursor population. Such a study may serve to improve our basic understanding of the osteoblast lineage. Theoretically, bioreactor tissue culture and use of any

type of antibody to markers diagnostic of cell change, would potentially be useful for analysis of differentiation and the cell arrangement achieved in morphogenesis.

In addition to cell differentiation, tissue morphogenesis is an important process. Very little is known about the arrangement and patterning of cells in the absence of gravity, or a low shear, simulated weightless environment, such as the bioreactor. Explant organ culture, such as the pre-metatarsal system described above, provides cells arranged within an intact three-dimensional extracellular matrix. This biological system can be combined with the rotating tissue culture system developed by NASA, particularly, the slow turning lateral vessel (STLV), and the high aspect ratio vessel (HARV). The combination of the bioreactor with the degree of specificity we can assign to sequential states in the mesenchyme to bone differentiation system will provide insight into 1) the advantages (or disadvantages) of simulated microgravity on differentiation and 2) the validity of the culture hardware devices as models of microgravity.

CONCLUDING COMMENTS

The future looks bright for ultimate tissue culture studies in bioreactors, aboard space station *Freedom,* that can provide clues to greater degrees of understanding of gravity in bone development. Culture studies have the potential for applying the microgravity environment to a biomedical solution of bone, and other tissue disorders, here on earth. As the opportunity for basic biological and biomedical research expand within the field of microgravity, the depth of understanding of fundamental cell and tissue processes will increase immensely. We are presently planning experiments using this embryonic pre-metatarsal developing system for analysis during space shuttle flights which are anticipatory to bioreactor studies on earth and on orbit.

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REFERENCES

Bagi C, Burger EH Mechanical stimulation by intermittent compression stimulates sulfate incorporation and matrix mineralization in fetal mouse long-bone rudiments under serum-free conditions Calcif Tissue Int 45 342–347, 1989

Bourne G Phosphatase and calcification In Bourne GB (ed) "The Biochemistry and Physiology of Bone" New York Academic Press, Vol IIII, pp 79–120, 1974

Bruder SP, Caplan AI Discrete stages within the osteogenic lineage are revealed by alterations in the cell surface architecture of embryonic bone cells Connect Tissue Res 20 73–79, 1989

Bruder SP, Caplan AI Osteogenic cell lineage analysis is facilitated by organ cultures of embryonic chick periosteum Dev Biol 141 319–329, 1990

Burger EH, Veldhuijzen JP, Klein-Nulend J, VanLoon JJWA Osteoclastic invasion and mineral resorption of fetal mouse long bone rudiments are inhibited by culture under intermittent compressive force Connect Tissue Res 20 131–141, 1989

Burger EH, Klein-Nulend J, Veldhuijzen JP Modulation of osteogenesis in fetal bone rudiments by mechanical stress *in vitro* J Biomech 24 101–109, 1991

Garetto LP, Gonsalves MR, Morey ER, Durnova G, Roberts WE Preosteoblast production 55 hours after a 12 5 day spaceflight on Cosmos 1887 FASEB 4 24–28, 1990

Klein-Nulend J, Veldhuijzen JP, Burger EH Increased calcification of growth plate cartilage as a result of compressive force *in vitro* Arthritis Rheum 29 1002–1009, 1986

Klein-Nulend J, Veldhuijzen JP, vanStrien ME, deJong M, Burger EH Inhibition of osteoclast bone resorption by mechanical stimulation *in vitro* Arthritis Rheum 33 66–72, 1990

Klement BJ, Spooner BS Endochondral bone formation in embryonic mouse pre-metatarsals Kans Acad Sci Trans 95(1–2) 39–44, 1992

Klement BJ, Spooner BS Embryonic mouse pre-metatarsal development in organ culture J Exp Zool (in press)

Morey ER, Baylink DJ Inhibition of bone formation during space flight Science 201 1138–1141, 1978

Posen S, Neale FL, Clubb JS Heat characterization in the study of human alkaline phosphatase Ann Intern Med 62 1234–1243, 1965

Roberts WE, Mozsary PG, Morey ER Suppression of osteoblast differentiation during weightlessness Physiologist [Suppl]24 575–576, 1981

Spengler DM, Morey ER, Carter DR, Turner RT, Baylink DJ Effects of spaceflight on structural and material strength of growing bone Proc Soc Exp Biol Med 174 224–228, 1983

Turner RT, Bell NH, Duvall P, Bobyn JD, Spector M, Holton EM, Baylink DJ Spaceflight results in formation of defective bone Proc Soc Exp Biol Med 180 544–549, 1985

Vico L, Chappard D, Polle S, Bakulin AV, Novikov VE, Alexandre C Trabecular bone remodeling after seven days of weightlessness exposure (BIOCOSMOS 1667) Am J Physiol 255(Regul Integrative Comp Physiol 24) R243–R247, 1988

Wolf DA, Schwarz RP Analysis of gravity-induced particle motion and fluid perfusion flow in the NASA-designed rotating zero-head-space tissue culture vessel NASA Tech Paper 3143 1–12, 1991