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COOPERATIVE AGREEMENT PROGRAM NCC 9-36, ROUND II

COVER SHEET FOR FINAL REPORT

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IN-52
626071

Name of Subcontractor: Dr. Pauline (Jackie) Duke

Title: Associate Professor

Institution: The University of Texas Health Science Center at Houston

Name of Project: "Use of NASA bioreactor in engineering tissue for bone repair"

Amount of Grant: [redacted]

* Amount Spent, if Different from Amount Granted:

Date Project Was Completed: March 31, 1998

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"Use of NASA bioreactor in engineering tissue for bone repair"
Final Report

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D. Montufar-Solis - Res. Associate

This study was proposed in search for a new alternative for bone replacement or repair. Because the systems commonly used in repair of bony defects form bone by going through a cartilaginous phase, implantation of a piece of cartilage could enhance the healing process by having a more advanced starting point. However, cartilage has seldom been used to replace bone due, in part, to the limitations in conventional culture systems that did not allow production of enough tissue for implants (2). The NASA-developed bioreactors known as STLV (Slow Turning Lateral Vessel) provide homogeneous distribution of cells, nutrients, and waste products, with less damaging turbulence and shear forces than conventional systems. Cultures under these conditions have higher growth rates, viability, and longevity, allowing larger "tissue-like" aggregates to form, thus opening the possibilities of producing enough tissue for implantation, along with the inherent advantages of *in vitro* manipulations (3,4). To assure large numbers of cells and to eliminate the use of timed embryos, we proposed to use an immortalized mouse limb bud cell line as the source of cells.

I. Cell line characterization, propagation, preservation and recovery from freezing.

Cultures using the **MPLB-1** (mouse posterior limb bud-1) cell line were set up using T-flasks to test for cell line propagation, preservation and recovery from freezing. Results were positive, good cell aggregation and nodule formation was observed. Initial characterization indicated that differentiating cells formed cartilaginous nodules, as evidenced by histological staining for sulfated mucosubstances in the cartilage matrix.

Cultures using the **MPLB-2** cell line were also set up to run a comparative study with the MPLB-1 cell line. Results showed that MPLB-2 cells had an initially slower growth rate than MPLB-1, and that the phenotype is more of fibroblasts than of chondrocytes. Based on these results from cell line characterization, the MPLB-1 cell line was chosen to be used for growth inside the bioreactor to produce cartilaginous aggregates for implantation.

II. Growth of MPLB-1 cell line inside a bioreactor.

MPLB-1 cells were cultured inside a bioreactor with a culture vessel capacity of 110ml (Synthecon, Inc). To increase cell proliferation and reduce differentiation in the system in order to allow growth of larger aggregates, some cultures were grown without ascorbic acid. Cells were also cultured either with or without aggregation prior to injection to the rotating vessel; and with or without attachment to cylindrical agar molds (7mm L, 4mm dia.). Control cultures were maintained in T-flasks. The superior growth of cells within the bioreactor was immediately apparent. Results also showed that cells used in this application did not have to be aggregated or provided with a substrate prior to putting them in the system. After only 3 days of culture it was clear that the cells were proliferating faster in suspension than when attached to the agar templates. Aggregates were cultured for 107 days. The cells growing in suspension formed large size aggregates that were used for implantation at selected times, but the cells grown

attached to the agar templates did not proliferate as rapidly and did not form aggregates suitable for implantation.

Image analyses of nodule size, as indicated by area, perimeter, minimum and maximum diameter, and density showed that aggregates increased in size throughout the more than three month culture period. The mean area of aggregates within the culture increased during the course of the first two months. At the end of the third month, the area of the largest aggregate measured was $20mm^2$ and the smallest $.01mm^2$. There is continued formation of small aggregates from single cells that proliferate and form new growing groups of cells.

Aggregate staining with Alcian blue (pH=1) indicated the presence of cartilaginous matrix, but light microscopy of the aggregates grown without ascorbic acid showed cells with appearance of pre-chondrocytes with very little metachromasia. Thus, this was the basal condition of the aggregates grown without ascorbic acid upon implantation: not very well differentiated, although some cartilage matrix (metachromatic matrix) was present and sometimes even a perichondrium formed around the aggregates. With the addition of ascorbic acid, the basal condition of the aggregates upon implantation was slightly more differentiated, had more cartilage matrix, but had little hypertrophy still. This confirmed our predictions of being able to manipulate the cartilage phenotype through changing the medium components. However, the amount of hypertrophy observed in the aggregates, as well as the amount of matrix produced was not comparable to that obtained from primary cultures.

III. Aggregates implanted subcutaneously in mice.

Cartilaginous aggregates grown inside the bioreactor were implanted, subcutaneously, in the costal region of eight-week old, C3H male mice (Harlan Sprague Dawley). Appropriate animal care, consistent with the Animal Welfare Act and NIH Policy and Guide were provided throughout this experiment. Two weeks prior to implantation, mice underwent surgery to insert a glass disc in each costal region, in order to induce formation of a non-inflamed vascular connective tissue in the site of future implantation. At time of aggregate implantation, disks were removed and aggregates were implanted. Three groups of mice received implants of aggregates grown without ascorbic acid: 1) Aggregates grown for 41 days were implanted for 1-5 weeks. 2) Aggregates grown for 62 days were implanted for 1-9 weeks. 3) Aggregates grown for 84 days were implanted for 1-12 weeks. Aggregates grown with ascorbic acid for 24 and 32 days were implanted for 2d, or 3-5 weeks.

Aggregates grown inside the bioreactor and implanted for at least 3 weeks showed good vascularization. Hypertrophy, however, was limited even in those aggregates implanted at more advanced stages of differentiation. Results in the in vivo implant system do not seem to be as clearcut as when primary limbbud cells rather than a cell line was used. In some cases, the cells within the implants appear to have been multipotent, and yielded muscle as well as cartilage-like tissue. This result may be due to the lack of hypertrophy reached in the initial nodules that were implanted, as previous studies with primary cultures indicated that formation of bone might be dependent on the initial stage of differentiation of the implant.

IV. Fulfillment of specific aims.

Specific aims have been fulfilled as follows:

Aim 1. To assess the ability of MPLB2 (Mouse Posterior Limb Bud-2), a mouse limb bud cell line, to produce healthy cartilaginous aggregates of a particular size and shape when cultured in the commercially available STLV known as a RCCS (Rotary Cylindrical Culture System; Synthecon, Inc.). The MPLB-1 cell line produced large numbers of cells, which differentiated well in T-flasks, forming numerous Alcian-blue positive nodules. Growth in the bioreactor was also phenomenal, but little differentiation of cartilage in the aggregates in the bioreactors occurred.

Aim 2. To identify the stage and amount of cartilage within the aggregates prior to implantation, through assessment of the quality (normality) of the cartilaginous aggregates produced. MPLB-1 cell aggregates grown inside the bioreactor were large cartilaginous nodules, as assessed by cartilage specific Alcian blue staining. However, the stage of differentiation showed little hypertrophy and reduced amount of matrix. This results are similar to those previously observed in microgravity, in vivo and in vitro.

Aim 3. To quantitate the amount of bone produced by the implant in an in vivo implant system. Implanted nodules appeared to have vascularized, but cells within nodules were apparently multipotent and produced other tissue besides cartilage on implantation. These results have led us to return to our original system of culturing cells from mouse limb buds, in spite of the difficulty of having to use large number of timed pregnant mice.

V. Presentations/publications. (*Attachments.)

1. Lecture on "The Use of Engineered Cartilage to Repair Bony Defects" for a graduate course in Biomaterials at the UT Dental Branch, Oct. 23.
2*. Two presentations, with an abstract and one paper published in the proceedings of In Space '96 conference, Tokyo, Japan, Nov. 11-12, organized by the Japan Space Promotion Center (JSUP). Presented results of previous studies and current research, as well as potential uses of the bioreactor on Space Station. "The Differentiation of Chondrocytes in Altered Gravity" and "New Views, New Vistas in the Life Sciences: ISS and Beyond".
3*. Presentation at the 15th Annual Houston Conference on Biomedical Engineering Research. Abstract.
4*. Presentation at the 13th Annual Meeting of the American Society for Gravitational and Space Biology (ASGSB), Washington, DC, November 19-22, 1997.
5. Presentation at the Third Annual UT-Houston Research Day, Edwin Hornberger Conference Center, October 3, 1997.
6*. Presentation at the Second Conference on Recent Advances in Fermentation Technology (RAFT II), "Microgravity Fermentations" session, San Diego, California, November 15-18, 1997. Abstract.

TISSUE ENGINEERING IN SPACE: PRODUCING CARTILAGE FOR REPAIR OF BONES ON EARTH.

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NASA's interest in tissue engineering derived from the idea that it might be possible in space to grow tissues, for example, articular cartilage to cover a bone's surface, without distortion of the growing tissue by gravity. In implementing its tissue engineering studies, which fall under the biotechnology program of the Microgravity Science and Application Division, NASA has concentrated on development of a bioreactor which can be used at 1g, or in space, to grow three-dimensional tissues which are more representative of in vivo tissue construction. Tissues grown to date include articular cartilage and bone, as well as cocultivation of cancer cells and normal cells. Temporary cartilage (or endochondral cartilage), that is, cartilage that is replaced by bone, has also been cultivated because a serious problem facing the orthopedic or oral surgeon today is the procurement of materials to provide support and induce bone during repair of bony defects[1]. This problem is complicated by the loss of traditional cadaver sources of bone due to potential infection with HIV. Because many bones form by endochondral ossification, that is, by bone replacement of a cartilage model, which is also the process by which fractures heal, cartilage can be used to repair bone, but its use has been limited in part by the amount of tissue available from conventional tissue culture systems. Embryonic limb cells cultured in a Rotating Wall Vessel produce cartilage and the cartilaginous aggregates grown in this system can be implanted subcutaneously, where they hypertrophy, calcify, vascularize, and recruit additional cells. But on earth, gravity limits the size of aggregates that can be grown in this system. Therefore, we plan to use microgravity to engineer cartilaginous tissue that can be used in repair of bony defects. By using the Rotating Wall Perfused Vessel (RWPV) tissue culture system developed by NASA coupled with the microgravity environment we plan to grow pieces of cartilage tissue of a size that has not been possible on earth, a problem that has limited the use of cartilage in bone repair, despite its immunogenic privilege. To assure large numbers of cells and to eliminate the use of timed embryos, we propose to use an immortalized cell line as a source of cells, the MPLB1 cell line derived from embryonic mouse limb bud.

Supported by TMC/NASA Subcontract NCC9-36.

[1] Journal of Cellular Biochemistry 51 (3), 1993.

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Poster Presentations

1 **Evan G. Pivalizza**
E.G. Pivalizza, D.C. Abramson, F.S. King
Heparinase-Celite Thromboelastography in Liver Transplantation

2 **Evan G. Pivalizza**
E.G. Pivalizza, P.J. Pivalizza
Perioperative Sonoclot Analysis in Pediatric Patients

3 **Sara Dann**
Cynthia Chappell
S.M. Dann, B.M. Salameh, P.C. Okhuysen, H.L. Dupont, C.L. Chappell
Antigen Specificity of Fecal IgA from Cryptosporidium parvum Challenged Volunteers

4 **Sandra Hanneman**
S.K. Hanneman, H.T. Cozart
Subjective Nursing Assessment of Cough Effort Intensity

5 **Craig W. Johnson**
C.W. Johnson, A. J. Abedor, G. Oser
Lecture versus Web Based Instruction in Biostatistics

6 **Jeffrey I. Toward**
J. Toward
An Exploration of the Theoretical Basis for Exercise and Physical Activity Participation in Older Adults

7 **Jackie Duke, Dina Montufar-Solis**
J. Duke, D. Montufar-Solis
Tissue Engineering in Space: Producing Cartilage for Repair of Bones on Earth

Cartilage Grown in Space for Repair of Bones on Earth. P.J. Duke* and D. Montufar-Solis. University of Texas Health Science Center, Dental Branch. Houston, TX, 77225.
A problem facing the oral surgeon today is the procurement of materials to provide support and induce bone during repair of bony defects. Because many bones form by endochondral ossification, cartilage can be used to repair bone, but its use has been limited in part by the amount of tissue available from conventional tissue culture systems. The rotating wall systems developed by NASA provide homogeneous distribution of cells, nutrients, and waste products; conditions that promote higher growth rates, viability, and longevity that allow larger 3-D "tissue-like" aggregates to form. Using these vessels, we have grown cartilage aggregates that when implanted subcutaneously begin the ossification process. But on earth, the size of aggregates we can produce in this system is still limited by the presence of gravity which results in a constant need to increase the RPMs to maintain aggregates in suspension. The sedimentation effects can not be overcome without excessive rotation, thus subjecting cells to considerable forces. In space however, rotation is not required to keep aggregates in suspension, and their size will not be limited by sedimentation. The microgravity environment will allow us to grow pieces of cartilage tissue of a size that has not been possible on earth, large enough to be useful in bone repair. --Supported by TMC/NASA Subcontract NCC9-36.