THREE-DIMENSIONAL TRANSGENIC CELL MODELS TO QUANTIFY SPACE GENOTOXIC EFFECTS

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ABSTRACT

In this paper we describe a three-dimensional, multicellular tissue-equivalent model, produced in NASA-designed, rotating wall bioreactors using mammalian cells engineered for genomic containment of multiple copies of defined target genes for genotoxic assessment. The Rat 2λ fibroblasts (Stratagene, Inc.) were genetically engineered to contain high-density target genes for mutagenesis. Stable three-dimensional, multicellular spheroids were formed when human mammary epithelial cells and Rat 2λ fibroblasts were cocultured on Cytodex 3 Beads in a rotating wall bioreactor. The utility of this spheroidal model for genotoxic assessment was indicated by a linear dose response curve and by results of gene sequence analysis of mutant clones from 400μm diameter spheroids following low-dose, high-energy, neon radiation exposure.

INTRODUCTION

In order for the human exploration of space to continue, the genotoxic hazards that are encountered in this unique environment must be identified and elucidated. The spacecraft environment contains radiation and chemical agents known to be mutagenic and carcinogenic to man. Additionally, microgravity represents a complicating factor that may modify or synergize induced genotoxic effects. Exposure to this environment raises the prospects for significant biological consequences arising from radiation-induced damage at the genetic level. Currently, there is a need for a model test system that is representative of cellular interactions present in tissue, capable of quantifying genotoxic damage induced by low levels of radiation and chemicals, and able to extrapolate assessed risk to man.

Estimating the risks associated with such unique occupational exposure is somewhat problematic. Risk assessment, based on animal studies, is confounded by our current lack of basic knowledge of the differences in metabolism and genetic repair processes between species. While *in vitro* models have advanced our understanding of many aspects of genotoxicity, human cells are frequently not used, thereby making risk extrapolation to man more difficult. These *in vitro* models also have the propensity to overlook the dynamic effect of intercellular interactions within tissues. The sensitivity of these models may be significantly increased in cell culture systems that provide dynamic intercellular interactions and tissue morphogenesis, thus providing a more robust platform for assessing the outcome of exposure to multiple stressors. NASA-designed bioreactors have enabled the formation of three-dimensional tissue-like assemblies from normal and neoplastic cells, both on Earth (Tsao & Gonda, 1999; Akins et al., 1997, 1999; Becker, 1993 and Jessup, 1993) and in space (Freed, 1999).

The unique ability of these bioreactors to support multicellular, multilayer, three-dimensional tissue-like development has been attributed to a low fluid shear culture environment, the randomization of the

gravity vector and the colocalization of suspended cells, cell aggregates, and microcarrier beads in the culture medium (Tsao and Gonda 1999; Goodwin et al., 1993).

The simultaneous co-culture of two cell types has been shown to enhance growth. For example, a variety of human and murine cells packaged into spheroids of human HeLa tumor cells exhibited enhanced clonogenicity (Djordjevic and Lang, 1990). Differentiation has also been observed. Human keratinocytes grown with fibroblasts on nylon mesh showed pleiomorphic differentiation into the 4 layers that is characteristic of skin tissue (Fleischmajer et al., 1993). In addition, both tumor and normal cells have been shown to exhibit functions characteristic of their *in vivo* counterparts that are not observed in two-dimensional cultures. The growth of cells in NASA bioreactors has been shown to produce: [1] Mineralization, type I collagen biosynthesis, and three-dimensional growth in human and rat osteosarcoma cell lines (Gonda et al., 1991b; 1992); [2] Formation of contracting myotubes in satellite cells cultured on Matrigel-coated beads (Molnar et al., 1997). Co-culture of normal human stromal cells with human colon carcinoma cells produced morphological differentiation in the cancer cells (Jessup et al., 1993). Co-culture of human prostate cancer cells and normal prostate fibroblasts showed patterns of histogenesis characteristic of tumors and responded to signal induction for growth and differentiation (Zhau et al., 1997).

We describe here a novel, state-of-the-art, three-dimensional model test system capable of the identification and quantification of inherent genotoxic effects present in the spacecraft environment and provide initial results of ionizing radiation.

MATERIALS AND METHODS

Cells

The human mammary epithelial (H184B5F5M10) cells, passage 42-47, were cultured as monolayers in Modified Eagles Medium, alpha modified, containing 10% fetal calf serum and 5 mM Hepes. They were derived from human breast epithelial cells (Yang and Craise, 1994). These cells are immortalized, nontumorigenic, exhibit normal G_1/G_0 arrest upon obtaining confluency, and exhibit both anchorage dependent and serum-dependent growth (Yang et al., 1997). Normal repair of induced radiation damage has also been observed (Yang et al., 1989).

The Big Blue™ rat cell line passages 9-20, obtained from Stratagene, Inc. (Austin, TX), were cultured as monolayers in DMEM containing 5% FBS and 200 ug/ml geneticin. It was derived from a rat 2λ embryonic fibroblast cell line genetically engineered to contain the Big Blue λLIZ shuttle vector and the pSV2NEO plasmid (Wyborski, et al., 1995). Each cell contains approximately 60 copies of the λLIZ shuttle vector that contains the *LacI* and *αLacZ* genes enabling mutations to be quantified using the Big Blue™ chromogenic assay. The Big Blue cell line has been successfully used in numerous *in vitro* mutagenesis studies (Kohler et. al., 1991; Provost et al., 1993; Rogers et al., 1995).

Bioreactor

The NASA-designed High Aspect Ratio Vessel (HARV) bioreactor (Synthecon, Houston, TX) is a non-perfused, horizontally rotating bioreactor for low-shear suspension culture of cells and tissues in a fluid environment (Figure 1). The 50 ml bioreactor was inoculated with cells and Cytodex™ 3 beads and completely filled with medium. Bioreactors were placed in a $37^{\circ}C$, 5% CO_2 atmosphere, and 99% humidity incubator and the vessel rotated at a rate to maintain suspension of aggregates and spheroids.

Coculture

Rat 2λ fibroblasts and H184B5F5M10 human epithelial cells were conditioned to growth in DMEM + 10% FBS before addition to bioreactor. Cells were harvested with trypsin/EDTA, centrifuged at 1000 rpm and suspended in growth medium (Figure 2). On day 0, rat 2λ fibroblasts were inoculated into the HARV with Cytodex™ 3 beads (Pharmacia) at a concentration of 2.5×10^5 cells/ml and a cell/bead ratio of 20:1. Inoculated bioreactors were placed in a 37°C, 5% CO_2 atmosphere, and 99% humidity incubator and rotated at 12 RPM. Cocultures were initiated on day 6 by inoculating 5.0×10^5 epithelial cells. Bioreactors were monitored daily for glucose, pH, dissolved gases (pO_2 and pCO_2) and bicarbonate concentration. Cell attachment, growth, and aggregate formation were monitored by phase contrast microscopy.

Histochemistry

Specimens were washed with phosphate buffered saline (PBS), fixed with Omnifix™, washed 2x with PBS, and dehydrated through a graded alcohol series. After infusion with xylene, paraffin embedding and sectioning, specimens were deparaffinized and stained with hematoxylin/eosin or Movat.

Radiation Treatment

The frequency of induced mutation was determined in the transgenic Rat 2 λcells. Spheroids were exposed to 0, 0.25, 1, and 2 Gy heavy ion neon irradiation at the National Institute of Radiological Sciences in Chiba, Japan. The dose rate was 0.3 Gy/min for all doses with the exception of 2 Gy, which was 1.5 Gy/min. Spheroids were grown for 5 days to allow post-irradiation expression of mutations and then snap frozen in liquid nitrogen.

Mutation Analysis

The use of the *lacI* gene in the Big Blue mutagenesis assay has been well established with a large database for comparison and is very reproducible between different laboratories (Piegorsch et al., 1994; Rogers et al., 1995). Frozen spheroids were lysed and genomic DNA isolated using a Stratagene DNA Isolation Kit. The lambda transgenic shuttle vector (containing the *lacI* target gene) was recovered from the isolated DNA and packaged into viable lambda phage particles using Stratagene's Transgene™ *in vitro* Packaging Extract. The packaged phage particles were then allowed to infect and lyse *E. coli* to form plaques on a bacterial lawn. Blue colored plaques are indicative of mutations while nonmutant plaques are colorless. The mutant frequency is the ratio of blue plaques to the total number of plaques. Mutant plaques were cored from the agar plates and replated for verification and sequencing. DNA sequencing was done according to the method described by Erfle, H.L. (1996). This method has been widely used to determine changes in the mutation spectrum of the LacI gene following exposure to potential toxicants (Shane et al., 2000; Stuart et al., 2000.
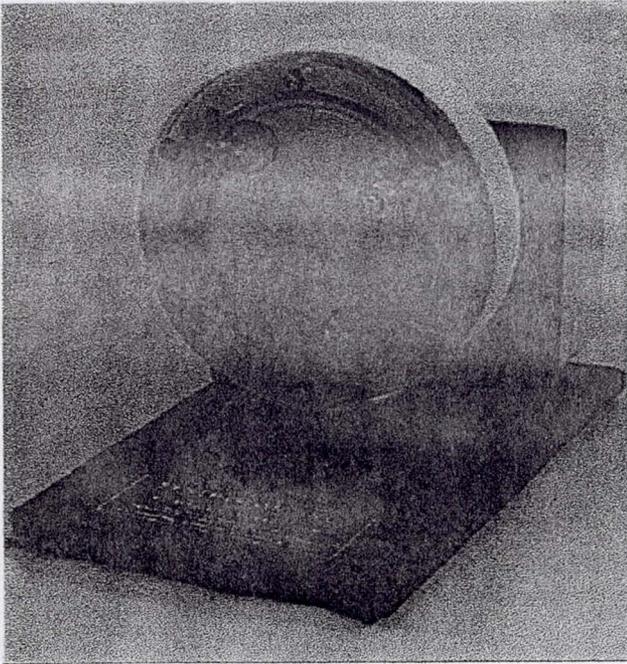
Fig. 1. The High Aspect Ratio Vessel (HARV) is a nonperfused, horizontally rotating bioreactor consisting of a 50 ml vessel having a large radius and a short length. The vessel is connected to a variable-rate motor and mounted on a fixed base. The vessel has several separate sample ports for adding media or reagents and removing samples. The HARV has been optimized for culturing suspension cells and anchorage-dependent cells with or without microcarriers.
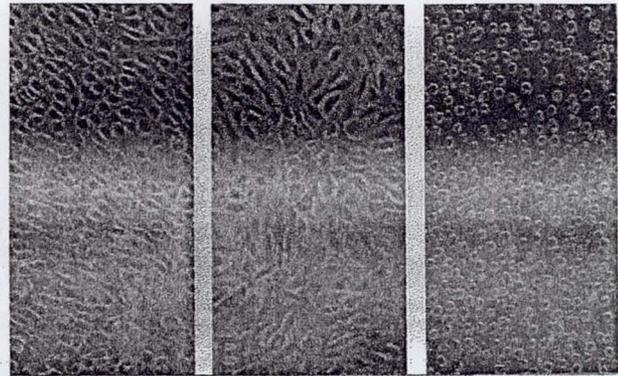

Fig. 2. Monolayer controls of (A) Rat 2λ fibroblasts and (B) Human epithelial cells cultured in tissue culture flasks. (C) Single cell starting inoculum of trypsinized Rat 2λ fibroblasts for the HARV bioreactor.
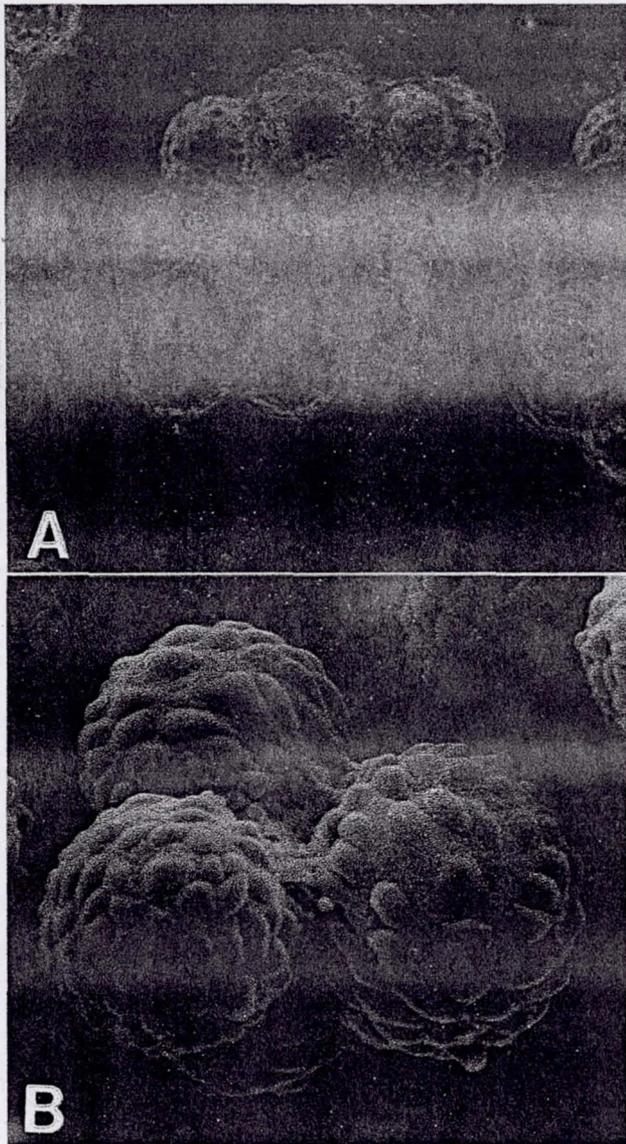
Fig. 3. Phase contrast and SEM photographs of three-dimensional aggregates of fibroblasts cultured on 150 μm diameter Cytodex 3 beads for 5 days in the HARV bioreactor. Note complete covering of individual beads by cells and dense cellular growth between beads. Coculture was

initiated at this time with inoculation of 5×10^5 epithelial cells/ml.
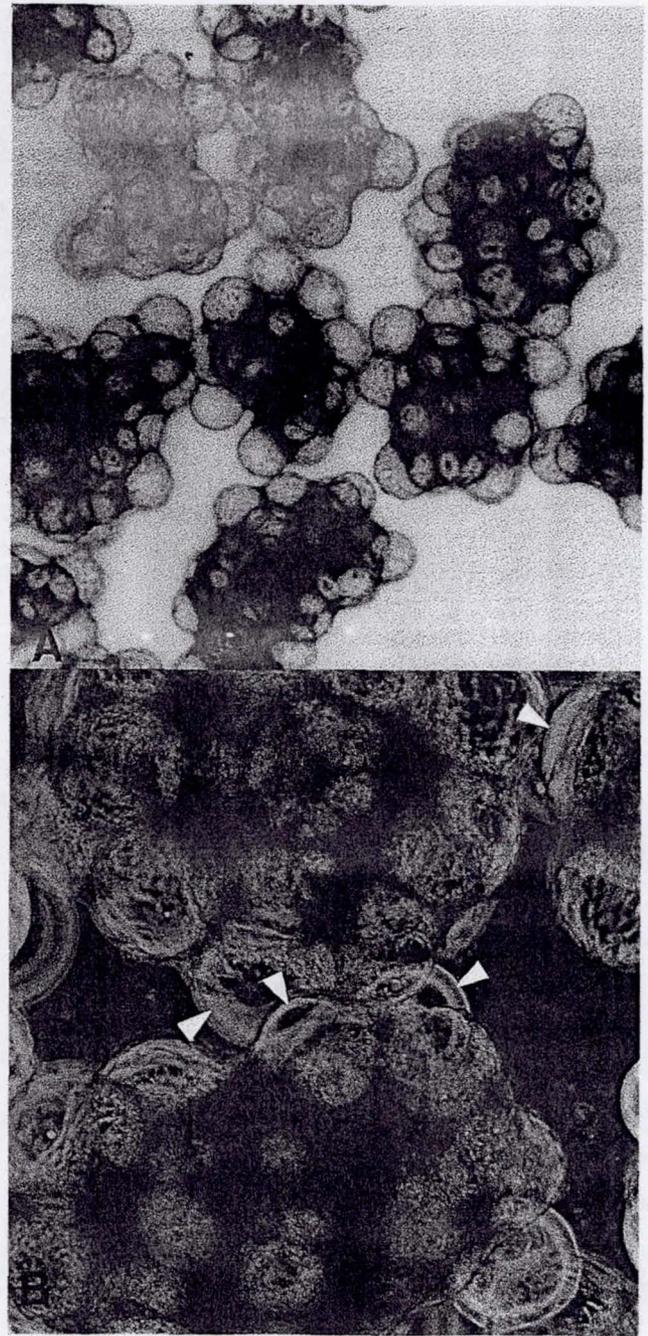

Fig. 4. Phase contrast micrographs of fibroblast and epithelial cells, cocultured in the HARV bioreactor with 150 μm diameter Cytodex 3

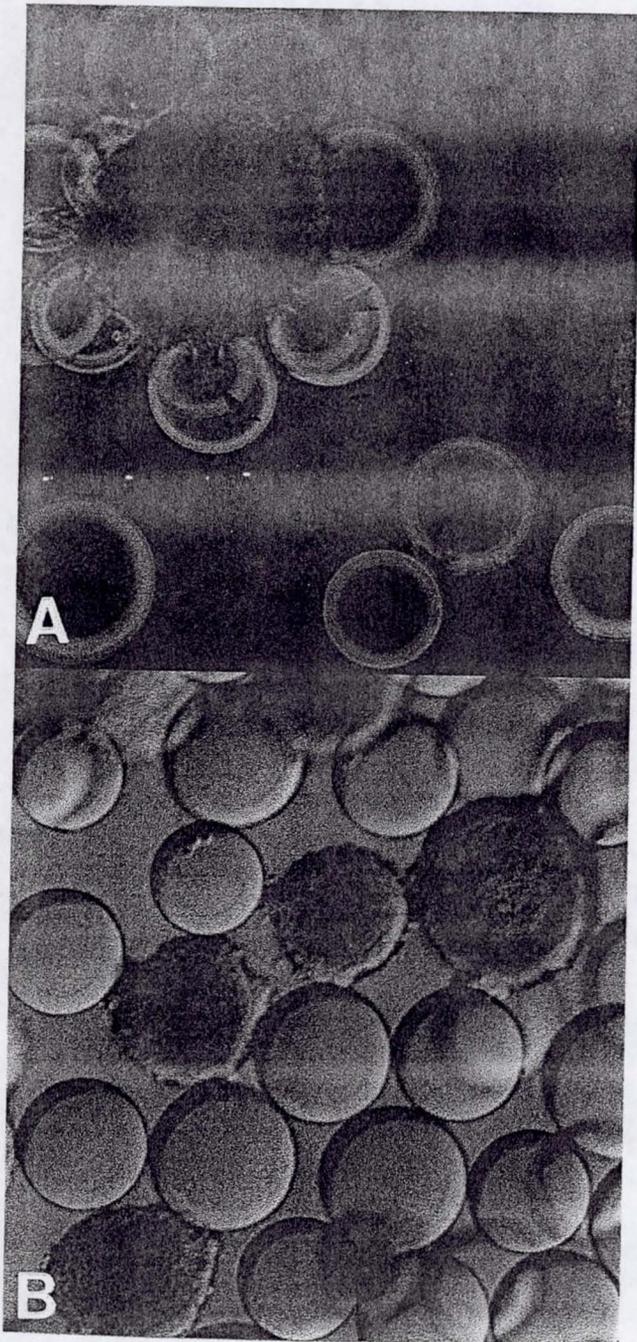beads for three days. Aggregates show dense intrabead cell growth and the beginning of retraction (arrows).

Fig. 5. Phase contrast micrographs of spheroids formed from the coculture of fibroblast and epithelial cells in the HARV bioreactor with 150 m diameter Cytodex 3 beads for eight days. (A) Final release of beads from spheroids (arrows). (B) Spheroid formation is completed with numerous free spheroids and "naked" beads.
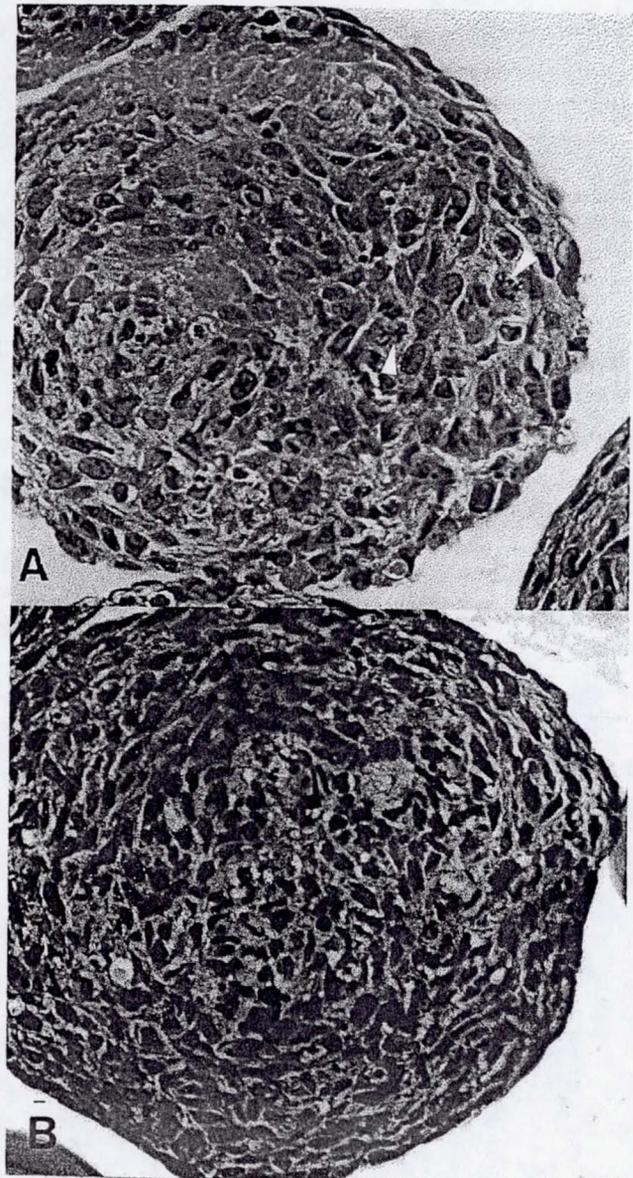
Fig. 6. Histological analysis of 14-day spheroids. Hematoxylin and eosin stained sections revealed an even cellular distribution and a low dividing fraction (1-2%) of cells throughout the spheroids (arrows). Movat stained spheroids show the formation of new collagen (yellow-green) by fibroblasts throughout the spheroid with some central areas containing greater deposition.
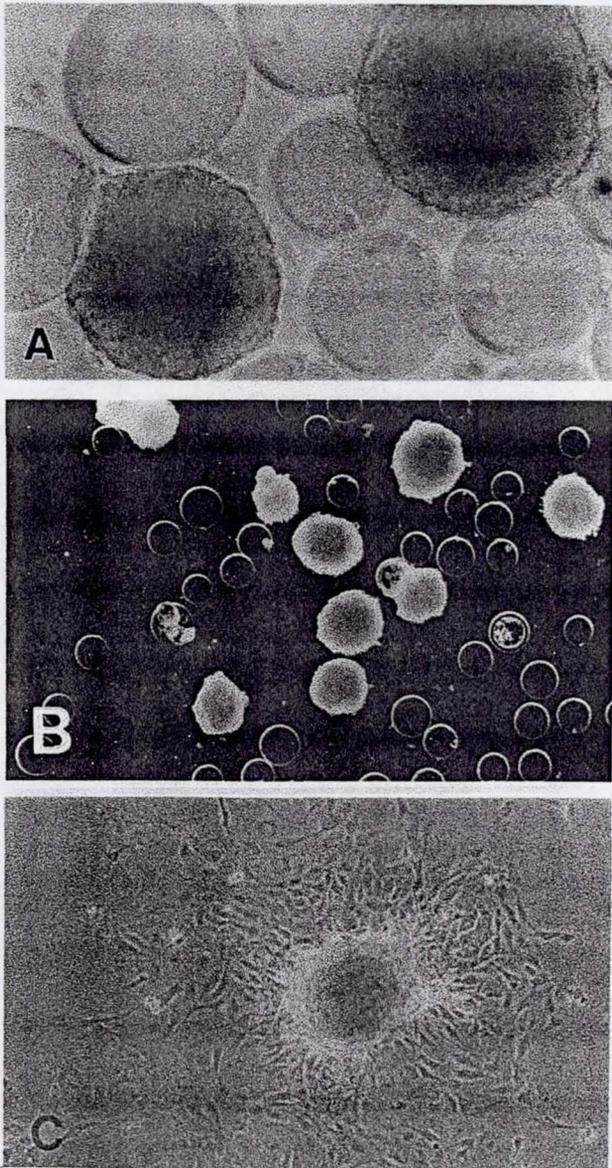

Fig. 7. Three-dimensional spheroids from 9-day coculture of fibroblasts and epithelial cells. (A) Complete release of spheroids from beads. (B) Multiple spheroids and free beads harvested from bioreactor. (C) Attachment and migration of cells from spheroids onto surface of tissue culture flask after 5 days.
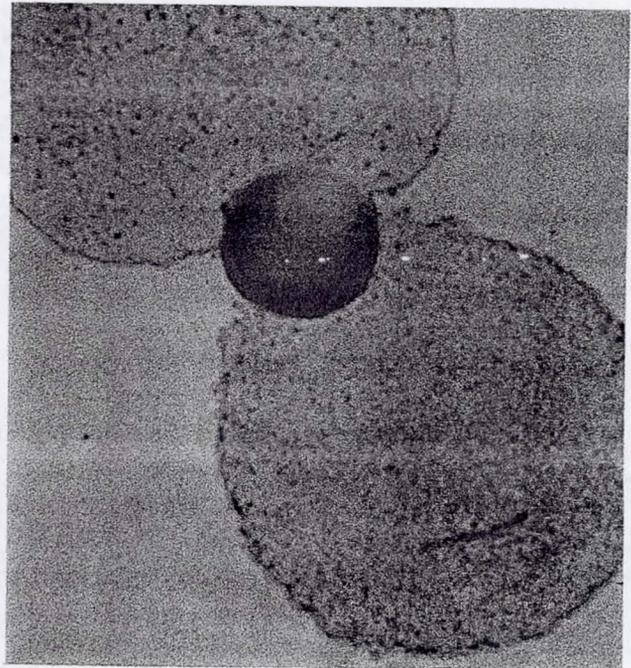

Fig. 8. 30-day spheroids maintained in Teflon culture bags for a period of 30 days and assessed for stability and viability by Trypan Blue staining. Note uptake of stain by free Cytodex 3 bead.

RESULTS & DISCUSSION

Spheroid Formation in Bioreactor

Fibroblasts readily attached to Cytodex 3 beads within hours of inoculation into the HARV bioreactor and completely covered the bead surface by day 4. Large three-dimensional multi-bead aggregates of fibroblast-covered beads with cells bridging between beads were present by day 5 (Figure 3). Large multibead aggregates with dense growth between and around the beads were readily observed 72 hours after addition of epithelial cells and by the eighth day of coculture, initial retraction of the cellular mass from the beads was observed (Figure 4). As retraction progressed, beads were continually released from the multibead aggregates eventually resulting in the appearance of free spheroids and numerous free "naked" beads beginning at day 14 (Figure 5).

Histology

Histological analysis by hematoxylin and eosin revealed a loose arrangement of cells within spheroids and a low dividing fraction of cells (1-2%) throughout (Figure 6). The formation of new collagen within an extracellular matrix was indicated in Movat stained sections by the presence of yellow-green staining throughout and strong staining within the center of the spheroids (Figure 6).

Spheroid Stability and Cell Viability

For assessment of inherent or induced genotoxic effects on long-duration missions, it is necessary for the cells in the test system to remain viable for long time intervals with minimal care. Trypan blue dye exclusion was utilized to assess gross viability of spheroids grown in the HARV bioreactor for 30 days and then placed into a 25 ml Teflon bag (frequently used for space experiments) for 30 days in a 37°C incubator. A portion of the culture medium was changed during the 30-day post-HARV period. Trypan blue exclusion revealed good viability of cells (>98%) within the spheroids (Figure 8). As a further evaluation of viablity, 30-day post-HARV spheroids were placed into a tissue

culture dish. The cells readily attached and grew out, confirming their reproductive viability (Figure 9). These results suggest that the cells in our spheroid model can remain viable for the prolonged periods required to support long exposures during space investigations.

Radiation-induced mutation

The effects of increasing radiation dose on mutation frequency using the *lacI* transgene are shown in Figure 9. The spontaneous background mutation rate averaged 3.7×10^{-5} and is equivalent to levels reported by Strategene. Treatment of spheroids with increasing doses of radiation resulted in a 4 fold induction of mutations ($15.4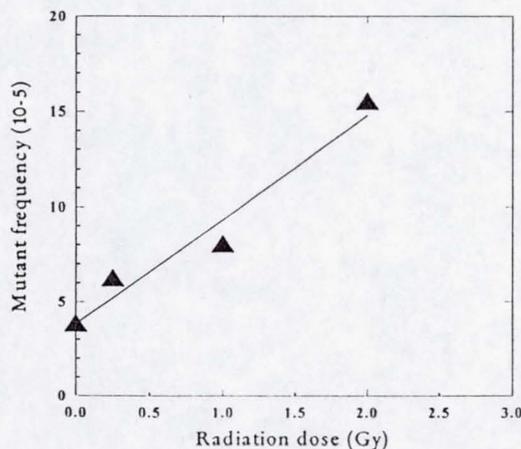 \times 10^{-5}$ at 2.0 Gy), as compared to the background control. Overall, there was a linear dose response for the frequency of mutations induced by increasing radiation dose.

Fig. 9. Radiation-induced mutation

Table 1. *lacI* **Gene Sequence Analysis**

Treatment	Mutation	Base	Amino Acid
Control	Substitution	G 575 C (3)**	Ala 183 Pro
	Substitution	G 56 A	Ala 10 Thr
	Substitution	T 162 A	Leu 45 Gln
	Substitution	G 287 C	Ala 87 Pro
	Substitution	C 92 G	Arg 22 Gly
	Substitution	G −15 T	0
	Substitution	G 701 C	Gly 225 Arg
	Substitution	C 92 T	Arg 22 Cys
Neon (0.25 Gy)	Substitution	G 575 C (2)**	Ala 183 Pro
	Substitution	G 56 A	Ala 10 Thr
	Substitution	G 53 T	Val 09 Phe
	Substitution	T 45 A	Leu 06 Stp
	Substitution	G −34 T	0
	Substitution	G 437 T	Glu 137 Stp
	Deletion	CC 795/6	—
	Deletion	G 389/91	—
Neon (1.0 Gy)	Substitution	G 575 C**	Ala 183 Pro
	Substitution	C 92 A	Arg 22 Ser
	Substitution	A 1004 T	Arg 326 Stp
	Substitution	G 525 A	Gly 166 Asp
	Substitution	A 101 T	Asn 25 Tyr
	Deletion	66 (-7bp)	—
	Deletion	348 (-1bp)	—
	Insertion	620 (+CTGG)	—

Table 1 shows the results of initial sequencing analysis of the *lacI* transgene from mutant

plaques recovered (cored) from a Big Blue assay. In the untreated control samples, only simple base substitutions were observed. Three control samples had the same mutation, indicative of a possible clonal expansion in the fibroblast cell line. This was further substantiated by the observation of the same mutation in irradiated samples. The detection of mutations resulting from clonal expansion allows more accurate calculation of the actual overall mutation frequency since these duplicates can be taken into account. The sample irradiated with 0.25 Gy Neon was found to contain two small deletions in addition to base substitutions. The sample irradiated with 1.0 Gy contained a simple deletion, a 7 base pair deletion, and a 4 base pair insertion, along with base substitutions. Although the data presented is limited, it suggests that a higher frequency of deletions and other multiple base sequence changes (i.e., insertions) occur with increasing radiation dose.

NASA-designed bioreactors were used to develop three-dimensional, tissue-like spheroids by coculturing human mammary epithelial cells and Rat 2 lambda fibroblasts. Histological analysis of the spheroids indicated a relatively uniform distribution of cells and a ECM. Immunohistochemical analysis using antibodies to vimentin and cytokeratin revealed an approximate 50:50 mixture of the two cell types distributed throughout the spheroids with fibroblasts concentrated toward the core of the spheroids and epithelial cells concentrated toward the periphery (data not shown).

Long-term spheroidal stability and cellular viability was established for long term space exposure. The utility of this spheroidal model for assessing the genotoxic effects of low-dose, high-energy ionizing radiation was initially tested with Neon exposure. A linear dose response curve and increasing numbers of deletions and insertions with increasing dosage was observed. Analysis of genotoxic effects of high energy iron ionization exposure at Brookhaven National Lab of both spheroids and fibroblast monolayers is underway. In addition, our epithelial coculture partner has recently been transfected with the Big Blue λLIZ shuttle vector and will be used to form

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spheroids containing both genetically engineered cell types for mechanistic and species-specific gentoxicity studies.

The three-dimensional spheroidal model containing genetically engineered fibroblasts is a model test system with the ability to: (i) Increase sensitivity through the analysis of multiple target sites of the same gene and/or multiple target sites comprised of different transgenes. (ii) Maintain spheroidal stability and cell viability with minimal care on long-duration space flight missions; (iii) Perform crucial mechanistic studies of the interactions of microgravity and radiation present in the space environment; and (iv) Measure multiple definitive endpoints; i.e., quantify mutation frequency, identify mutations at the sequence level, and assess induced chromosome aberrations.

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REFERENCES

Akins RE, Schroedl NA, Gonda SR, and Hartzell C. (1997) Neonatal rat heart cells cultured in simulated microgravity. In Vitro Cell Dev Biol-Animal 33:337-343.

Akins RE, Boyce RA, Madonna MI, Schroedl NA, Gonda SR, McLaughlin TA and Hartzell CR. (1999) Cardiac Organogenesis In Vitro: Reestablishment of Three-Dimensional Tissue Architecture by Dissociated Neonatal

Rat Ventricular Cells. Tissue Engineering. 5: 103-117.

Becker JL, Prewett TL, Spaulding GF, and Goodwin TJ. (1993) Three dimensional growth and differentiation of ovarian tumor cell line in high aspect rotating-wall vessel: morphological and embryologic considerations. J Cell Biochem 51:283-289.

Djordjevic B and Lange CS. (1990) Clonogenicity of mammalian cells in hybrid spheroids: a new assay method. Radiat Environ Biophys 29:31-46.

Erfle HL, et al. (1996). An efficient laboratory protocol for the sequencing of a large number of lacI mutants recovered from Big Blue transgenic animals. Environ. Mol. Mutagen. 28:4:393-396.

Fleischmajer R, MacKonald ED 2, Contard P and Perlish JS. (1993) Immunochemistry of a keratinocyte-fibroblast co-culture model for reconstruction of human skin. J Histochem Cytochem 41:1359-1366.

Freed LE, Langer R, Martin I, Pellis N, and Vunjak-Novakovic G. (1997) Tissue Engineering of Cartilage in Space. Proc. Natl. Acad. Sci. 94:13885-13890.

Gonda SR, Marley GM, Sinha R, Joseph T. and Tuan RS. (1991) Three-dimensional spheroid formation and mineralization of cultured osteosarcoma cells in a simulated microgravity environment. J Cell Biol 115:450a.

Gonda SR, Marley GM, Schroedl NA, Tuan RS, Sinha RK, Joseph T. and Ingram M. (1992) NASA Bioreactor: a unique in vitro fluid culture system for three-dimensional tissue formation with anchorage-dependent cells. In Vitro Cell Develop Biol. 28:4A.

Goodwin TJ, Prewett TL, Wolf DA and Spaulding GF. (1993) Reduced shear stress: A major component in the ability of mammalian tissues to form three-dimensional assemblies in simulated microgravity. J Cell Biochem 51:301-311.

Jessup JM, Goodwin TJ and Spaulding GF. (1993) Prospects for use of microgravity-base bioreactors to study three-dimensional host-

tumor interactions in humans neoplastic. J Cell Biochem 51:290-300.

Kohler SW, et al. (1991). Spectra of spontaneous and mutagen-induced mutations in the *lacI* gene in transgenic mice. Proc. Natl. Acad. Sci. 88:18:7958 -7962

Molnar G, Schroedl NA, Gonda SR and Hartzell CR. (1997) Skeletal muscle satellite cells cultured in simulated microgravity. In Vitro Cell Dev Biol-Animal 33:386-391.

Piegorsch WW, Lockhart AC, Margolin BH, Tindall KR, Gorelick NJ, Short JM, Carr, GJ, Thompson ED and Shelby MD. (1994) Sources of variability in data from a lacI transgenic mouse mutation assay. Environ Molec Mutagen 23:17-31.

Provost GS, Kretz PL, Hamner RT, Matthews CD, Rogers BJ, Lundberg KS, Dycaico MJ and Short JM. (1993) Transgenic systems for *in vitro* mutation analyais. Mutat Res 288:133-149.

Rogers BJ, Provost GS, Young RR, Putman DL and Short JM. (1995) Intralaboratory optimization and standardization of mutant

screening conditions used for a lambda/lacI transgenic mouse mutagenesis assay. Mutat Res 327:57-66.

Shane BS. et al. (2000) LacI mutation spectra following benzo (a) pyrene treatment of Big Blue mice. Carcinogenesis. 21:4:715-725.

Stuart GR. et al. (2000) Prostate mutations in rats induced by the suspected human carcinogen 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine. Cancer Res. Vol. 60:2:266-268.

Tsao YD and Gonda SR. (1999) A new Technology for Three-dimensional Cell Culture: The Hydrodynamic Focussing Bioreactor. 1999 American Society of Mechanical Engineering 44:37-38.

Tsao YD, Goodwin TJ, Wolf DA and Spaulding GF. (1992) Responses of gravity level variations on the NASA/JSC bioreactor system. The Physiologist 35:S49-S50.

Wyborski DL. et al. (1995). Development of a rat cell line containing stably integrated copies of a lambda/lacI shuttle vector. Mutation Research 334:2:161-165.

Yang TC, Stampfer MR and Tobias CA. (1989)
Radiation studies on sensitivity and repair of
human mammary epithelial cells. Int J
Radiat Biol 56:5:605-609.

Yang CH and Craise LM. (1994) Development
of human epithelial cell systems for radiation
risk assessment. Adv Space Res 14:10:115-
120.

Yang TC, George KA, Craise LM and Durante
M. (1997) Initiation of oncogenic
transformation in human epithelial cells by
charged particles. Radiat Oncol Invest 5:134-
138.

Zhau HE, Goodwin TJ, Chang SM, Baker TI and
Chung LWK. (1997) Establishment of a
three-dimensional human prostate organoid
coculture under microgravity-simulated
conditions: evaluation of androgen-induced
growth and PSA expression. In Vitro Cell
Dev Biol-Animal 33:375-380.