Cellular Responses to Mechanical Stress

Selected Contribution: A three-dimensional model for assessment of in vitro toxicity in *Balaena mysticetus* renal tissue

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**Goodwin, T. J., L. Coate-Li, R. M. Linnehan, and T. G. Hammond.** Selected Contribution: A three-dimensional model for assessment of in vitro toxicity in *Balaena mysticetus* renal tissue. *J Appl Physiol* 89: 2508–2517, 2000.—This study established two- and three-dimensional renal proximal tubular cell cultures of the endangered species bowhead whale (*Balaena mysticetus*), developed SV40-transfected cultures, and cloned the 61-amino acid open reading frame for the metallothionein protein, the primary binding site for heavy metal contamination in mammals. Microgravity research, modulations in mechanical culture conditions (modeled microgravity), and shear stress have spawned innovative approaches to understanding the dynamics of cellular interactions, gene expression, and differentiation in several cellular systems. These investigations have led to the creation of ex vivo tissue models capable of serving as physiological research analogs for three-dimensional cellular interactions. These models are enabling studies in immune function, tissue modeling for basic research, and neoplasia. Three-dimensional cellular models emulate aspects of in vivo cellular architecture and physiology and may facilitate environmental toxicological studies aimed at elucidating biological functions and responses at the cellular level. Marine mammals occupy a significant ecological niche (72% of the Earth’s surface is water) in terms of the potential for information on bioaccumulation and transport of terrestrial and marine environmental toxins in high-order vertebrates. Few ex vivo models of marine mammal physiology exist in vitro to accomplish the aforementioned studies. Techniques developed in this investigation, based on previous tissue modeling successes, may serve to facilitate similar research in other marine mammals.

**bowhead whale; environmental toxicology; modeled microgravity; rotating-wall vessel; physiology**

**INTEREST IN PERSISTENT ENVIRONMENTAL pollutants, such as heavy metals, organochlorines, and chlorinated pesticides, and their possible effects on marine and terrestrial mammals has existed for many years. Tissue levels of such pollutants are documented in numerous marine mammal species, including *Balaena mysticetus* (i.e., the bowhead whale) (3, 5, 30, 32, 34). In the bowhead whale and the walrus, increasing levels of cadmium have been reported (2, 4, 44). In the bowhead whale, the highest levels of cadmium are in the renicule (kidney), with the most elevated concentrations found in older and larger (i.e., by body length) whales. Previously, Henk et al. (21) studied the renicule morphology of the bowhead whale in some detail. Establishing the age of bowhead whales is difficult, but a recent study indicates that longer whales are indeed older (13). Therefore, extrapolation of body morphometry at time of harvest allows for approximation of age and the ability to assess the accumulation of heavy metals in bowhead renal tissues over time.**

Many heavy metals are toxic to the mammalian physiology; however, some arctic marine species (e.g., bowhead whale, walrus, and ringed seal) seem to tolerate tissue levels that impair the function of or are lethal to terrestrial mammals (mouse, rat, humans).
(11). With respect to mercury, the beluga whale accumulates very high concentrations complexed in association with selenium and silver (2). Therefore, mercury accumulation has been hypothesized as a metallothionein (MTH)-independent process that is strongly linked with selenium (much different from that in terrestrial species) (2, 22). This implies that a species that can tolerate higher tissue levels of cadmium and/or mercury is likely to have a protective mechanism. If such protective mechanisms could be reasonably understood, there might be ways to render less tolerant species, such as humans, more resistant to the toxic insult of heavy metals. This would have obvious applications for humans subjected to closed environmental systems, such as spacecrafts and lunar and Martian habitats, for extended periods. Manned travel to other planets will require long-duration flights and thus the recycling of resources within these closed systems. Therefore, the National Aeronautics and Space Administration (NASA) is interested in studying natural mammalian systems that can resist environmental pollutants such as heavy metals. The apparent ability of large (old) bowhead whales to tolerate high tissue levels of heavy metals and the ready access to fresh tissue from subsistence-harvested bowhead whales has prompted this initial investigation to determine whether bowhead whale tissue, and perhaps other marine mammal tissue, might serve as a model for resistance to the toxic effects of heavy metals. An investigation of this type requires the development of robust bowhead whale cell cultures and organized tissues in the laboratory. Bowhead whale cells were grown in tissue culture on at least two earlier occasions: one pertaining to establishment of the karyotype (23) and the other pertaining to recovery of adenoviral isolates 80B1 and 80B7 (38–40). These studies, however, did not result in robust cultures. Obtaining long-term in vitro cultures of bowhead whale tissues is important in that methods would be applicable for study of other cetaceans and marine mammals (e.g., beluga and pilot whales, walruses, and seals) that exhibit increased levels of environmental contamination.

During the past decade, significant progress has been made in utilizing NASA’s rotational technology (rotating-wall vessel (RWV)) to optimize high-fidelity, low shear stress, low-turbulence, three-dimensional bioreactor cell culturing for multiple biological investigations of normal and neoplastic tissues in a variety of mammalian systems (15, 16, 18, 20, 24, 27, 47). In each of these studies, successful three-dimensional, high-fidelity, differentiated, ex vivo models were created. Recently, the RWV system was used to develop a tissue model of the bowhead whale for potential environmental toxicology investigations (14).

The term RWV encompasses a family of vessels, batch-fed and perfused, that embody the same fluid dynamic operating principles. These principles are 1) solid body rotation about a horizontal axis, characterized by the simultaneous achievement of a) collocation of particles of different sedimentation rates, b) extremely low fluid shear stress and turbulence, and c) three-dimensional spatial freedom; and 2) oxygenation by active or passive diffusion to the exclusion of all but dissolved gases from the reactor chamber, yielding a vessel devoid of gas bubbles and gas-fluid interface (i.e., yielding zero headspace) (37, 46).

The horizontally rotating culture vessel simulates some aspects of microgravity (hence the terminology “modeled microgravity”) (9, 36, 37, 40) and reduces to a minimum the shear and turbulence associated with impeller-driven, stirred bioreactors. Microcarriers and cells remain uniformly suspended in the fluid, provided that the optimal rotational speed is applied. Shear forces and mass transfer in solid body rotation are attributed to the minute movements of the microcarriers in the medium and their incidental contact with the wall and one another. Conventional methods of oxygenation disturb the medium and damage the cells (7, 8). Previous designers suspended particles in a quiescent environment by horizontal rotation (e.g., Vaseen, US Patent 4,223,094, 1980), but none was able to provide a means of oxygenation that did not perturb the tranquil environment. Many stirred bioreactors depend on gas control and diffusion at the gas-medium interface (i.e., headspace oxygenation). Many others use sparging or airlift techniques in which gas is introduced as minute bubbles that lift the cells and microcarriers up through the medium. All these methods damage fragile cell-to-cell interactions. In contrast, RWV technology incorporates gas diffusion through a silicone membrane. All batch-fed RWVs use this method of oxygenation and use a centrally located silicone membrane for transfer of dissolved gases (37). Incubator air is circulated through the center shaft of the vessel by means of an external air pump, which eliminates the induction of air bubbles into the culture medium and produces an environment conducive to fragile cell assembly.

In view of these facts, a project is underway to accomplish the following objectives: 1) collect viable cellular samples of renal tissue from subsistence-harvested bowhead whales, 2) establish robust bowhead whale two-dimensional renal cell cultures, 3) establish three-dimensional cell cultures (i.e., modeled microgravity) of bowhead whale renal cells utilizing RWV technology, and 4) subject this three-dimensional model to analysis to characterize the culture and to assess the cellular responses and plasticity for transfection.

MATERIALS AND METHODS

Renal cell culture and transfection. Renal samples reported here were collected from several bowhead whales, one harvested in the spring of 1997 (97B7) and seven harvested in the fall of 1998 (98B10, 98B12, 98B14, 98B15, 98B17, 98B19, and 98B20) during subsistence hunts at Barrow, Alaska. Tissues (150–200 g) were removed and placed into a plastic bag with sterile antimiycotic-antibiotic medium and cooled on ice until arrival at the Arctic Research Facility at Barrow. Tissues were rinsed twice with high concentration (3×) fungicide-antibiotic PBS and then washed twice with complete GTSF-2 medium. GTSF-2 growth medium was developed in the Biotechnology Laboratories at NASA’s John-
son Space Center (Houston, TX) to accommodate the nutritional needs of fastidious cell cultures. Briefly, the medium is formulated on a tri-sugar base containing glucose, galactose, and fructose in a mixture of 40% MEM-a and 60% L-15 Leibovitz medium with 6% FCS (18, 27); this medium has proven valuable in the establishment of primary cell cultures. Connective tissue, fat, and excess tissue were removed by dissection. Tissues were prepared for several explicit investigations (i.e., cell culture, isolation of RNA for MTH analysis, and archiving viable cells and tissue). All tissue preparations included multiple washes with PBS, as described above, to remove as many red blood cells as possible before tissue disposition. Three-quarters of each sample was sliced into thin, wafer-sized 1.5- to 2.5-mm-diameter medallions. Several medallions were homogenized in a Waring blender, using gentle pulsation, until a fine slurry was produced. The slurry was explanted into T75 flasks with GTSF-2 (18, 27) and placed in a 37°C incubator with 10% CO₂ to begin incubation. This material was later hand carried to Johnson Space Center. For RNA preservation, medallions were diced into 1- to 3-mm sections, placed into cryopreservation vials, and quick frozen in liquid nitrogen. Tissue samples for cryopreservation and explant initiation were finely diced and placed in 10% DMSO-20% FCS in complete GTSF-2 medium and stored in liquid nitrogen. The remaining one-quarter of the tissue was also diced into 1- to 3-mm pieces. Half of this material was placed in 3% glutaraldehyde-2% paraformaldehyde in PBS for scanning electron microscopy analysis, and the other half was minced even more finely and preserved with 3% glutaraldehyde for immunohistochemical analysis.

**Cell transfection.** Renal cell tissue explants 98B10 and 98B12 were designated G98B10K and G98B12K, respectively, before being transfected for immortalization by the method of Chen and Okayama (6). Briefly, exponentially growing cells were trypsinized, seeded at 5 × 10⁵ cells per 10-cm plate, and incubated overnight in 10 ml of GTSF-2 growth medium. Each cell line was inoculated with 20–30 μg of plasmid SV2 Neo or SV3 Neo (American Type Culture Collection, Manassas, VA) DNA and mixed with 0.5 ml of a 0.25 M CaCl₂ solution and then added to 0.5 ml of a 2× concentration of BBS (Sigma Chemical, St. Louis, MO). The mixture was incubated for 10–20 min at room temperature with hood lights off. Calcium phosphate-DNA solution (1 ml) was added dropwise to the plate of cells and swirled gently. Samples were incubated for an additional 4–24 h at 35–37°C under 2–6% CO₂. Medium was removed, and the cells were rinsed twice in the growth medium, refed, and incubated for 24 h at 35–37°C under 5–6% CO₂. Cells were subcultivated at (>1:10) and incubated for an additional 24 h before stable transformants were selected. Because of the extreme scarcity of the tissue, varying temperatures and CO₂ ranges were used in the initial definition of appropriate transfection conditions for these tissues. Optimal parameters are reported in the Results.

**Transfection efficiency.** Transfected cells were subcultivated and plated at 10⁵ cells per 10-cm plate 24 h before selection. For each transfection, a total of four plates of 10³ cells were used: two plates for selection and two for a plating efficiency assay. To determine the number of Neo⁺ transformants and the optimal G418 concentration, cells were selected for 2–3 wk in GTSF-2 growth medium containing varying concentrations (100–600 μg) per milliliter of G418 growth medium. Transformation efficiency, expressed as percent transformation, was calculated by dividing the average number of colonies (>100 cells) by the average number of colonies that grew in nonselective growth medium and then multiplying the result by 100. The transformation frequency of the cells was determined as follows. After transfection, as described above, cells were plated in a 24-well dish at 100 cells per well and selected in G418 medium (100–600 μg/ml) for 3 wk. The number of wells containing proliferating cells was assessed. Transformation frequency was calculated by dividing the number of positive wells by the total number of cells selected. Plating efficiency was determined by maintaining the transfectants in growth medium for 1 wk and then staining and counting the colonies. Transfected cell cultures were verified by immunohistochemical staining with a mouse monoclonal antibody (working dilution 1:250) generated against SV40 large T cell antigen (Chemicon International, Temecula, CA).

**RWV culture conditions and hardware description.** As previously stated, these horizontally rotating culture vessels simulate some aspects of microgravity (9) by reducing to a minimum the shear and turbulence normally associated with impeller-driven stirred bioreactors. Designed with no internal moving parts, the vessel described here operates in an unusually low shear regime (0.2 dynes/cm²). Mixing and shear forces in this culture vessel result from the microcarrier motion in the medium and the contact that the microcarriers occasionally make with the wall and each other. These forces are minimal compared with the shear forces in stirred bioreactor systems (17).

The RWV used in these experiments was designed as outlined in US Patent 4,988,623. Cultures were initiated into the RWV by the method of Goodwin et al. (15–17, 47). Briefly, the vessel was completely filled with GTSF-2 medium (zero headspace) inoculated through a syringe port with Cytodex-3 microcarriers (average size is 125 μm). The vessel was then inoculated with renal cells at a concentration of 10 cells/microcarrier. Microcarrier concentration was 5 mg/ml (4,000 beads/ml), yielding ~2 × 10⁵ cells/ml. Vessel rotation was initiated at 18 rpm for the first 24 h to allow appropriate cell attachment to the microcarriers. Thereafter, the vessel was surveyed every 24 h for pH and O₂, CO₂, and glucose concentrations, and a 67% medium change was accomplished to replenish nutrients. Vessel fluid dynamics in solid body rotation were described previously (36, 37, 41, 46).

**Histology.** Kidney pieces were sectioned into 1- to 2-μm-wide longitudinal strips and fixed by immersion in Streck fixative (a proprietary reagent; Streck Laboratories, Omaha, NE) for 24–48 h. Tissues were rinsed in 50% and 70% alcohol and embedded in paraffin using a 7-h cycle. Serial 5-μm-thick sections, including all layers, were mounted on poly-L-lysine-coated glass slides, dewaxed with xylene and alcohol, and exposed to Inhibisol (1-1-1-trichloroethane) for histochemical analysis. Sections were stained with hematoxylin and eosin, periodic acid-Schiff, or Mason’s trichrome.

**Analysis of the proximal tubule epithelial markers.** Cellular enzymes were labeled for flow cytometry analysis on a cell-by-cell basis, as previously described by Dolbeare and Smith (12). To measure γ-glutamyl transpeptidase, leucine aminopeptidase, and alkaline aminopeptidase activities, γ-Glu, l-Leu, and 1-Ala derivatives of 4-methoxy-β-naphthylamine (4-MNA; Enzyme System Products, Livermore, CA) were used. The enzymes specifically cleave these substrates, liberating free 4-MNA. In the presence of 5-nitrosalicylaldehyde (5-NA) at pH 6.0, free 4-MNA is almost instantaneously trapped and precipitated. The product of 4-MNA and 5-NA is fluorescent in the visible spectrum (excited at 488 nm) and displays a broad emission spectrum from 510 to 680 nm (14, 19, 20), which facilitates simple flow cytometry analysis.
Flow cytometry analysis of cells and membranes. Flow cytometry analysis was performed on a FACS Vantage flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) using a dedicated PowerMac computer (24). Excitation was at 488 nm using a Coherent 5W argon-ion laser. For each particle, emission was measured using photomultipliers at 530 ± 30 and 585 ± 26 nm. Data were collected as 2,000-event list-mode files and analyzed with LYSYS software.

**MTH cloning.** RNA was isolated from frozen bowhead whale kidney, as described by Hammond et al. (20) and Kaysen et al. (24). Briefly, bowhead whale kidney was frozen in liquid nitrogen and ground to a powder in a mortar and pestle. TRIzol (Life Technologies, Rockville, MD) was then added to the powdered tissue, and total RNA was isolated. First-strand cDNA was transcribed using superscript reverse transcriptase and oligo(dT) primer. The MTH open reading frame was amplified using the forward primer based on the closest consensus sequence of known mammalian MTH primers, including the flanking regions of the open reading frame MTH PCR primers (AATGGACCCCAACTGCTG) and (GCMCAACAGTTGCACTTS). The PCR product was subcloned into the pGEM-EZ vector (Promega, Madison, WI) and then sequenced using the fMol cycle sequencing kit (Promega). The results were compared with those in GenBank using BLAST (National Center for Biotechnology Information, Bethesda, MD).

**RESULTS**

**Renal cell culture and transfection.** Arctic sample collection and isolation procedures resulted in the development of several cell lines of normal and transfected bowhead renal cells that are in robust culture in our laboratories. Development of these cultures was a nontrivial effort because of the extreme conditions of harvest and the variable lengths of time required to recover tissue after an animal was killed. In addition, the structure and composition of the reniculi of this marine mammal exacerbated the dissection and recovery of renal proximal tubular cells.

For identification purposes, the cell lines were given the previously described designations (see MATERIALS AND METHODS). Bowhead renal cells from whale 97B7 (female, 13.2 m long) were successfully grown in the laboratory. Cultures remain viable and are being propagated to densities that will allow further analyses. Light microscopy and trypan blue dye exclusion for viability have been performed on each of the cell cultures. To date, of the seven whales sampled in Fall 1998, successful renal cell growth was achieved for 98B10 (female, 13.1 m long), 98B12 (female, 11.3 m long), 98B14 (male, 8.2 m long), 98B19 (male, 9.4 m long), and 98B20 (male, 11.8 m long). Each of the cultures has been expanded and banked as a future resource.

**Transfection and plating efficiency.** Renal cell lines G98B10K and G98B12K were transfected with the large T cell antigen fraction of SV40 viral DNA, as described above. Successful transfections from these two cell lines were subsequently labeled T2G98B10K (Fig. 1A) and T3G98B12K (Fig. 1B), respectively. Figure 1C illustrates the negative control obtained by deletion of the primary antibody to SV40. Nontrans-
formed and transformed cells were continued in parallel cultures. Plating efficiency of the nontransfected cell lines from liquid nitrogen recovery was ~80–90%. Plating efficiency after optimized transfection at 36.5°C in 6% CO₂ and selection in 400 µg/ml of G418 was 70–85% for both transfected cell lines (i.e., T2G98B10K and T3G98B12K). Each of the transfected cultures exhibited approximately the same robust growth (1:10 passage at 6-day intervals), which persisted in continuous culture for at least 67 passages. Small samples of the native and transfected cells were cryogenically preserved for future experimentation. Samples of normal renal cultures began to show senescence at approximately passages 20–25.

Two of the cell lines described above were used to initiate successful three-dimensional cell cultures. Cell lines G98B10K and T3G98B12K were used to develop the three-dimensional models (Fig. 2). Each of the lines grew well as a three-dimensional model; cell growth covered the microcarriers and organized into tissue masses. Relatively little morphological change was seen between normal and transfected cells (Fig. 2). The ridges on the surfaces of both normal and transfected renal cells seem to be a physiological occurrence, and, although we do not know the function of such ridges, they do not appear to be common in renal tissues of terrestrial mammals.

Histology. In an attempt to assess the relative levels of current environmental heavy metal contamination in these tissues, we compared our 1997/1998 sections with histological sections of renal tissue taken from subsistence-harvested bowhead whales of similar length (female, 10.9 m long, and female, 10.8 m long; sections were courtesy of Dr. William G. Henk, Louisiana State University, Baton Rouge, LA; see Ref. 21 for more information). Figure 3 shows sections of bowhead whale tissue from 1985 (Fig. 3, A and B) and 1997/1998 (Fig. 3, C and D) stained with Masson’s trichrome. Here, an increase in fibrosis is shown, indicated by the red-purple fibrils in the contemporary bowhead whale.

![Fig. 2. Scanning electron photomicrographs show robust growth of 3-dimensional normal (A, B, and C) and SV40-transfected (D and E) renal proximal tubular cells developed from cell lines G98B10K and T3G98B12K, respectively. Compared with a microcarrier without cellular coverage (F), normal (A and B, cell line G98B10K) and transfected (D and E, cell line T3G98B12K) cells in 3-dimensional culture have covered the microcarriers and organized into tissue masses. Relative morphological changes between normal and transfected cells were minimal. Magnification is specified on each image.](http://jap.physiology.org/Downloaded/fromhttp://jap.physiology.org)
sections. This finding was consistent throughout the block and on blocks from several different whales. In addition, cellular damage is evident at the wall within the tubular lumen, as indicated by the irregularity of the intercellular junctions and morphological structures (Fig. 3, C and D vs. A and B). Although cellular damage and fibrosis were observed, neither was a factor in the development of the two- or three-dimensional cultures of bowhead renal cells because healthy cells were isolated to initiate the respective laboratory cultures.

Flow cytometry. Bowhead renal cells were analyzed before transfection with SV40 viral DNA. The proportion of proximal tubular cells in whale renal cell fractions was assayed using an entrapped fluorogenic substrate for the proximal enzyme markers γ-glutamyl transpeptidase, leucine aminopeptidase, and alanine transpeptidase as previously described (12, 19). Flow cytometry.
cytometry analysis on a cell-by-cell basis showed that bowhead renal cortical cells were 98 ± 2% \((n = 2)\) proximal tubules, as determined by flow cytometry analysis of aliquots for the proximal markers using Schiff base trapping of cleavage products of \(\gamma\)-Glutamyl 4-MNA and similar derivatives of \(\gamma\)-leu and \(\psi\)-Ala (12). Figure 4, A and B, depicts the distribution of 2,000 measurements on individual cells.

To confirm that the flow cytometry measurements were performed on cells and not dye crystals, the analysis of enzyme markers was repeated to correlate enzyme activity with particle size. Figure 4, C–E, depicts 2,000 cells with enzyme probe fluorescence, side scatter (an index of size), and the number of particles per channel. Display of the side scatter shows that cells were measured and not gating on crystals of precipitated dye, which would be far smaller than cells. Hence, the purity measurements are on cell populations (Fig. 4, C and D) and not a crystal artifact. The mixing study (Fig. 4E) shows that we can discriminate populations of cells from artifacts resulting from dye leakage between cells.

The cell cultures from 98B12 and 98B20 contain at least 92% renal proximal tubular cells. Tissue stains for \(\gamma\)-glutamyl transpeptidase, leucine aminopeptidase, alanine aminopeptidase, and cathepsin B indicated conclusively distinct populations of the renal proximal tubular cells.

**MTH cloning.** The RNA was transformed into first-strand cDNA, and the bowhead whale MTH sequence

Fig. 4. Flow cytometry analysis of 2,000 measurements on individual renal proximal tubular cells from a representative animal (A and B). A and B: curves represent unstained cells (1), cells stained with 5-nitrosalicaldehyde (5-NA) alone (2), and cells stained with \(\gamma\)-Leu-4-methoxy-\(\beta\)-naphthylamine (4-MNA) alone (3). A: unstained and stained cells as in B (curves 1–3) and cells stained with both the \(\gamma\)-Leu-4-MNA substrate and the 5-NA trapping agent (4). Repeat analysis of enzyme markers correlated enzyme activity with particle size for 2,000 unstained cells (C), 2,000 cells stained with both \(\gamma\)-Ala-4-MNA and 5-NA (D), and a mix of unstained cells and \(\gamma\)-Ala-4-MNA + 5-NA-stained cells (E). The number of particles per channel extends upward (C–E), and side scatter on the horizontal axes (C–E) demonstrates that cells, and not gating on crystals precipitated by dye, are being measured.
was determined. Forward primers based on the homologous regions of other known mammalian MTH sequences allowed us to use PCR and to sequence the entire open reading frame of the bowhead whale MTH sequence. The bowhead whale MTH sequence (Fig. 5) was submitted to GenBank using the National Center for Biotechnology Information online BankIt software and received the accession number AF022117.

**DISCUSSION**

*Two- and three-dimensional cell cultures.* This study demonstrates that primary renal cells can be successfully harvested in a subsistence-hunt setting, established in tissue culture, transfected, developed into two- and three-dimensional models suitable for physiology and toxicology investigations (Figs. 1 and 2), and banked for the study of an endangered species. Development of complex three-dimensional in vitro cultures of the bowhead whale serve to open for the first time the research vista for ex vivo modeling of several protected and endangered marine mammals known to be contaminated with heavy metals and other environmental toxins (22, 23, 35). Interestingly, ridged modeling seen on the surface of normal and transfected renal cells (Fig. 2, B and C) is a physiological occurrence of cells not previously observed by our research group in any other terrestrial mammal. Current studies are underway to discover whether this phenomenon is isolated to the bowhead whale or whether it is common to other marine mammals. Transfection of these tissues will enable researchers to conserve these valuable animal tissues liberated from subsistence hunting and to establish additional models for further investigations of the bowhead whale and other marine mammals. This opportunity provides a tool for the assessment of the cellular and molecular effects of environmental toxins ex vivo in an isolated system.

*Fibrosis.* Analysis of renal tissues taken from animals of similar relative age and size (length) reveal varying but increased levels of fibrosis in whales harvested between 1997 and 1998 compared with whales harvested in 1985. Bowhead whales and other marine mammals have a documented rise of heavy metal and other environmental toxins in kidney and liver tissues (4, 31, 33, 42, 43), and recently acquired bowhead kidney specimens indicate an increase in fibrosis (Fig. 3, C and D). One result of heavy metal accumulation is fibrosis, possibly brought on with increasing age, although this is far from the only cause of renal scarring. Scarring is commonly attributed to other causative agents, such as atrophy, inflammation, and parasitism (25, 33). Editorials have celebrated the resistance of arctic marine mammals to levels of cadmium, mercury, and other heavy metals that are toxic in terrestrial mammals (26, 31). Literature review, however, suggests that marine mammal physiology is able to complex mercury and selenium in the form of mercuric selenide (25, 26, 29), which may act as a protectant to toxic effects. Therefore, we suggest that the current fibrotic data are an impetus for further examination of the potential for heavy metal accumulation and the perceived ability of marine mammals to resist this insult.

*Flow cytometry.* Staining with an entrapped fluorogenic substrate for the proximal enzyme markers γ-glutamyl-transpeptidase, leucine aminopeptidase, and alanine transpeptidase, as described previously (12, 19), conclusively demonstrates the presence of an almost pure population (>92%) of renal proximal tubular cells. These data reinforce the applicability of the proposed study by substantiating the modeling of the cells directly involved in renal processing and MTH interaction.

*MTH.* The major transport protein for several heavy metals, which are abundant in the Arctic and global environments, is MTH (4, 31–33, 42–45). Although dozens of MTH sequences have been cloned in different species, according to the National gene bank (GenBank) and Huang et al. (22), there are no data for marine mammals. This study documents the mRNA and translated protein sequences of bowhead whale MTH (Fig. 5). The bowhead whale MTH sequence has all mammalian features and most closely matches the bovine MTH sequence (22). The mammalian features observed in the bowhead whale MTH sequence include 61 amino acids and a carboxy-terminal alanine. Clones of marine MTH sequences, compared with mammalian sequences that the bowhead sequence lacks, are characterized by 60 amino acids; carboxy-terminal glutamine or asparagine, where arginine or asparagine replaces lysine at residue 30 of the interdomain sequence CKKSCCSCCP, and proline can replace serine.

**Fig. 5.** Sequences of mRNA (A; GenBank accession number AF022117) and protein and metallothionein (B) of *Balaena mysticetus* (bowhead whale). MTH, metallothionein.

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**Bowhead mRNA sequence**

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| 1  | atggacccca actgcttctg cgcgcgagt ggatcttgca ctgtgscgg ctcttgcaaa |
| 61 | tgcaaggct gcaaaacc ccctctggag aagcagctgc gctctgcgct cccccccggc |
| 121| tgacaaaggt tgtgccaggg ctgctgtgca aaaggccct cccacaagtg caactgttg |
| 181| gca |
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**Protein sequence**

- **Bowhead**
  - MDP-ODCTCAAGDSCCAGSCKKNRCRS-CRSSCCCPACNNCAKVCYKPCAPSKK
- **Bovine**
  - MDP--CCEKSTGCSCCAGSCKSNACTSCFKEKGCCDCPSCSCSKACGVCYK--KCDT
- **Sheep**
  - MDP--NCCPTGCSCCAGSCKNAKPCSCS-CSSCCCPAGCAKACGVCYK--ASDKC
- **Chicken**
  - MDP--NCCCAAGGSCCAGSCKKECKKTCSCFSCCSCCPAGCTCAAGVCYK--ASDKC
- **Trout**
  - MDP--NCCPTGCSCCAGSCKNAKPCSCS-CSSCCPDAGCAKACGVCYK--ASDKC
at position 35 of the same sequence, with up to 14 serines by replacement of proline, lysine, glycine, alanine glutamine, and/or aspartic acid at various sites and at least three lysine residues replaced by either threonine or asparagine. The mammalian sequences are distinct from avian clones, characterized by features such as 63 amino acids, with carboxy-terminal lysine or histidine, where arginine or asparagine replaces lysine at residue 30 of the interdomain sequence CKKSCCSCCP, and up to 14 serines by replacement of proline, lysine, glycine, alanine glutamine, and/or aspartic acid at various sites. Hence, the bowhead whale MTH clone has all the molecular characteristics of a true mammal, and there are no unusual structural features of the bowhead MTH sequence to suggest that their metabolic ability for heavy metals is different from that of other mammals.

In conclusion, NASA’s involvement and the microgravity applications of this research may seem slightly odd until one realizes that, in addition to a charter to explore the cosmos, NASA also has a program entitled “Mission to Planet Earth.” This program is designed to observe, investigate, and intervene into issues concerning global ecology. The development of new technologies and mechanisms to analyze and study our planet and the potential impact on the global ecology to terrestrial and marine life are of paramount importance.

Current literature is replete with accounts of contamination in marine mammals worldwide (1, 10, 28, 33, 35, 43, 45). Through the efforts of these researchers and many others, examples of global pollution in our marine mammal populations are being documented. With the establishment of robust tissue cultures of the bowhead whale kidney, development of sophisticated three-dimensional analogs ex vivo, and molecular genetic definition of the bowhead MTH protein, we have the capability to investigate within the laboratory cellular physiological functions and molecular responses to environmental contaminants in a widening number of marine mammal populations; knowledge about closed environmental habitats in the microgravity, marine, and terrestrial environments can be advanced while conserving resources.

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