



ELSEVIER

Aquatic Toxicology 67 (2004) 195–202

**AQUATIC
TOXICOLOGY**

www.elsevier.com/locate/aquatox

Generation and partial characterization of a transformed cetacean cell line

M. Pine^a, M. Schroeder^b, K. Greer^a, R. Hokanson^a, D. Busbee^{a,c,*}

^a Department of Anatomy and Public Health and Center for Environmental and Rural Health, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843, USA

^b Department of Comparative Sciences, College of Veterinary Medicine, University of Wisconsin, Madison, WI 53706, USA

^c Department of Occupational and Environmental Health, School of Rural Public Health, Health Science Center, Texas A&M University, College Station, TX 77843, USA

Received 22 September 2003; received in revised form 16 December 2003; accepted 2 January 2004

Abstract

A primary epithelial cell line, DK1, established from renal tissue of a spontaneously aborted female Atlantic bottlenose dolphin was transfected with linearized pSV3.neo, an SV40 virus-derived plasmid encoding large tumor antigen (Tag). Transfected cells were grown in cetacean culture medium supplemented with 400 µg/ml geneticin (G418), and individual clones were selected using cloning rings. DKN1 was the first clone to be evaluated for future research use, and has been continuously cultured for 8 years. Intracellular cytokeratin and the expression of Tag were determined in DKN1, and cell growth was evaluated under different concentrations of L-glutamine, glutathione, and N-acetylcysteine. DKN1 cells did not require high levels of L-glutamine as previously reported for cetacean cells, and addition of antioxidants at the concentrations used in this study (2.0 mM) decreased the rate of cell division. These data suggest strongly that these immortalized bottlenose dolphin epithelial cells have different levels of, and requirements for, glutathione than would be considered normal for terrestrial mammalian cells, do not require high levels of L-glutamine as previously suggested for dolphin cells, and exhibit decreased levels of cell growth and viability in high levels of the antioxidant GSH and its precursor, NAC.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Bottlenose dolphin; Cetacean cell line; Transformed cell line; Reactive oxygen species; Glutathione; SV40 large T antigen; N-acetylcysteine; L-Glutamine

1. Introduction

Bottlenose dolphins, *Tursiops truncatus*, are carnivorous marine mammals normally found in relatively stable, site specific, populations that inhabit shallow

coastal and estuarine areas (Scott et al., 1990). These upper trophic level mammals are exposed to environmental chemicals, derived from agricultural and industrial contamination and metropolitan runoff, that tend to bioaccumulate up through the food chain and exist at highly variable concentrations in fatty tissues of the animals (Kuehl et al., 1991; Finklea et al., 2000). Specific mass dolphin mortalities have occurred worldwide in the last two decades and have been correlated

* Corresponding author. Tel.: +1-979-845-2828/6463; fax: +1-979-847-8981.

E-mail address: dbusbee@cvm.tamu.edu (D. Busbee).

with exposure to and bioaccumulation of environmental pollutants, some of which have subsequently been found to be potentially immunosuppressive (Aguilar and Borrell, 1991; Kuehl et al., 1991). While these mass mortalities have been shown to have a primary viral etiology, the potential for immunosuppressive chemicals contributing to viral disease must be considered. Among the chemical classes reported to be found in dolphin tissues are a number of halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs) (Finklea et al., 2000).

Many of the deleterious effects produced by anthropogenic compounds, such as the HAHs and PAHs, in addition to causing mutations, are believed to be due, in part, to the generation of free radicals and/or alterations in the reactive oxygen scavenging systems that protect eukaryotic cells from free radicals (Almeida et al., 1997). Further, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a prototypical organochlorine (OC) with known toxic effects, has also been reported to increase superoxide production (Hassoun et al., 1998) and cause a persistent oxidative stress response in exposed cells and tissues (Shertzer et al., 1998).

Although studies of contaminant bioaccumulation in tissues of bottlenose dolphins and other cetaceans have clearly shown the presence of potentially damaging chemicals, the effects of such chemicals on the physiology of these animals have resisted evaluation. In vivo physiological assays are essentially impossible to conduct on protected marine mammals, and cell lines that afford the opportunity to study kidney cellular effects over long time periods have generally not been available. A primary epithelial cell line established from kidney tissue of a spontaneously aborted female bottlenose dolphin in captivity was initially reported by Carvan et al. (1994). This cell line, originally named Carvan dolphin kidney (CDK) and later changed to dolphin kidney 1 (DK1), was partially characterized showing it to be epithelial in origin, to express the Ah receptor, and to have an apparently normal diploid chromosomal number of $2N = 44$. Additional studies using this cell line showed that it could be induced for CYP1A1, and that in an induced state the cells metabolized benzo(a)pyrene (BaP) forming BaP-DNA adducts and initiating DNA excision repair (Carvan et al., 1995). Further studies reported a banded karyotype with an ideogram of the karyotype,

and showed a remarkable degree of chromosomal homology between *Tursiops truncatus* and *Homo sapiens* (Bielec et al., 1997, 1998).

In this study, we report the transfection of DK1 cells with a linearized plasmid, pSV3.neo, to form an immortalized cell line (DKN1) that expresses the Simian virus 40 large tumor antigen (Tag). This cell line has been maintained for 8 years through a large number of cell divisions. Because the original cells were maintained in an enriched medium including three times the standard concentration of L-glutamine, we evaluated growth of the transformed cells under standard glutamine concentrations and under the enriched conditions believed to be required by dolphin cells. Additionally, the medium (CCM) was supplemented with two antioxidant compounds: reduced glutathione (GSH) and its synthetic precursor *N*-acetylcysteine (NAC), both of which have been shown to be protective of many cell types under conditions of oxidative stress. Our results indicate that DKN1 cells do not require additional L-glutamine supplementation and the addition of antioxidants, GSH and NAC, had a detrimental effect on cell growth.

2. Materials and methods

2.1. Cells

A primary dolphin kidney cell line, DK1, established in this laboratory (Carvan et al., 1994) was maintained in Dulbecco's Modified Eagle's Medium and Ham's F12 (GIBCO, Grand Island, NY) in a 1:1 ratio (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 10 mM NaCl, 10 ml/100× penicillin-streptomycin-neomycin (PSN) or 50 mg/l gentamycin, 2.5 µg/ml amphotericin B (Amp B), 3× minimal essential medium (MEM) amino acids including L-glutamine (9.0 mM), 3× MEM non-essential amino acids, and 3× vitamins. This modified medium, cetacean culture medium (CCM) was previously shown in this and other laboratories to greatly enhance the viability and cell division rate in primary cultures of cetacean fibroblasts and epidermal keratinocytes.

The SV40 transformed cell line, 2RA, derived from the human fetal lung fibroblast cell line, WI38, was obtained from the American Type Culture Collection

(ATCC). A homogenate of 2RA was prepared to use as a positive control for Tag expression in DKN1 cells as described by Srivastava et al. (1993).

A polyclonal hybridoma cell line (PAB101) secreting antibodies against different epitopes of SV40 large T antigen (Tag) was purchased from the ATCC and grown in roller bottles at 37 °C in DMEM containing 10% FBS, 0.1 mg/ml gentamycin sulfate (Sigma) and 0.625 mg/ml amphotericin B (Sigma).

2.2. Transfection

DK1 cells were transfected by electroporation with 1.0 µg of the linearized SV40-derived plasmid, pSV3.neo (a gift from Dr. Rodney Nairn, University of Texas System Cancer Center Science Park, Smithville, TX). After electroporation, the cells were plated at 2.5×10^3 cells/cm² into 60 mM Corning culture dishes with 2.0 mM grids and grown in CCM plus 400 µg/ml geneticin (G418) for selection of transfected clones. Cultures for selection were maintained at 37 °C in a humidified incubator with an atmosphere of 95% air and 5% CO₂. The medium was changed every third day. Individual clones were selected using cloning rings. From these, the DKN1 cell line was obtained as a clone for future research.

2.3. Anti-tag monoclonal antibody preparation

Anti-tag polyclonal antibody was purified from the medium of PAB101 cells by ammonium sulfate precipitation with subsequent passage over a protein A-Sepharose column (Biorad) according to established protocols (Current Protocols in Immunology 49 pp. (2.6.1–2.6.4)). Culture supernatant was collected by centrifugation at $400 \times g$ for 15 min at room temperature, filtered (0.46 µm), mixed at a 55:45 ratio of supernatant to saturated ammonium sulfate solution, and precipitated overnight at 4 °C. The precipitate was collected by centrifugation at 11,500 rpm in a Ti 13.1 rotor for 60 min at 4 °C, resuspended in cold phosphate buffered saline (PBS), applied to a protein A-Sepharose column, and eluted with 0.1 M sodium citrate, pH 4.5. Elution fractions were neutralized with Tris (pH 9.9). Peak fractions were determined using the A 280 values derived from a BSA/elution buffer standard curve and verified on a 10% SDS-acrylamide gel according to a previously

described protocol (Current Protocols in Molecular Biology 50 pp. (10.2.1–10.2.18)).

2.4. SV40 large tumor antigen evaluation

Simian virus 40 large tumor antigen (Tag) expression was evaluated by dot blot analysis of purified Tag, DKN1 cell preparations, and transformed WI38 (2RA) cell preparations using murine anti-Tag (PAB101) polyclonal antibodies and anti-mouse IgG monoclonal antibodies as described in Srivastava et al. (1993). Aliquots containing 10 µl of Tag or cell preparation supernatants were diluted with Tris buffered saline (TBS) containing 10 mM Tris-HCl (pH 8.0) and 150 mM NaCl. The diluted proteins were spotted onto nitrocellulose membranes for dot blot immunoanalysis detection of Tag (Srivastava et al., 1993). The negative control contained 1 mg/ml BSA in 50 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, 1 mM DTT, 1 mM EDTA, and 15% glycerol.

2.5. Cytokeratin detection by immunofluorescence

DKN1 cells were evaluated for the presence of cytokeratin using mouse anti-cytokeratin, mouse anti- α smooth muscle actin, mouse anti-desmin, and/or mouse anti-vimentin all from Sigma, St. Louis, MO. Briefly, cultures were fixed in -20 °C methanol, washed with 0.3% Tween 20 in PBS, blocked in 5% normal goat serum, incubated overnight at 4 °C with 2.0 µg/ml primary antibody, and detected with fluorescein-conjugated goat anti-mouse secondary antibody. Slides were overlaid with a coverglass and Prolong antifade mounting reagent (Molecular Probes, Eugene, OR).

2.6. Growth curves

DKN1 cells were seeded at a density of 5×10^3 cells per well in six-well plates. Treatment groups for each cell line were divided into two categories: CCM with a final concentration of 9.0 mM L-GLN designated CCM9 and CCM with a final concentration of 2.5 mM L-GLN designated CCM2.5. In each of these categories cells were grown in the presence of 2.0 mM reduced glutathione (GSH) purchased from Boehringer Mannheim, Germany or 2.0 mM *N*-acetylcysteine obtained from Sigma, St. Louis, MO. Every 48 h over a

2-week period, three wells were harvested with trypsin and counted separately using an improved Neubauer hemacytometer. Cell viability was determined using the Trypan Blue exclusion assay. Medium for each group was changed every 48 h throughout the study.

2.7. Statistical analysis

All treatment points are expressed as the mean of three separate cell counts plus or minus the standard error of the mean (S.E.M.). Means were compared using the analysis of variance (ANOVA) procedure with the computer program SAS version 8e. Any treatment point with a *P*-value of less than 0.05 was considered to be statistically significant.

3. Results

To evaluate the results of DK1 transfection, preparations of transfected cells were analyzed to determine whether or not they expressed the SV40 large tumor antigen, Tag. This would be the anticipated result of transfection of the linearized plasmid and would be expected to result in immortalization of this cell line. In this study we compared purified Tag, a cell preparation of WI38 cells, and a cell homogenate of transfected DK1 cells (DKN) using monoclonal antibodies developed against purified Tag. Transfected cells showed reactivity against anti-Tag antibody, indicating that they expressed the SV40 Tag (Fig. 1). These cells were subsequently named DKN1.

The original DK1 cells were shown to be epithelial in origin (Carvan et al., 1994). DK1 cells transfected using pSV3.neo, DKN1, were also suggested to be epithelial as evidenced by the presence of intracellular cytokeratin (Fig. 2) shown by immunofluorescence staining. These were compared with DK1 cells stained for intracellular cytokeratin (Fig. 2B). Although a different fluor-labeled immunoprobe was used to evaluate DK1 cells, both DK1 and DKN1 showed cytokeratin staining characteristic of epithelial cells.

Growth rates and patterns for DKN1 cells cultured in the original formula of CCM which contained high (9.0 mM) levels of L-glutamine were compared over a 14-day interval to DKN1 cells cultured in medium containing 2.5 mM L-glutamine (Fig. 3, panel A). These two culture media were designated CCM9

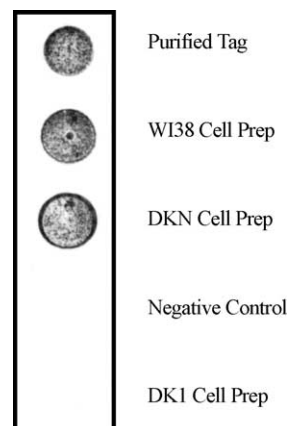


Fig. 1. Evaluation of the presence of the SV40 Large T antigen in transfected DK1 cells. The expression of SV40 derived large T antigen was evaluated in DKN cells using dot blot analysis. Preparations from transfected DK1 cells were compared to purified SV40 large tumor antigen protein, SV40 virus transformed WI38 (2RA positive control) cells, untransfected DK1 cells and to a negative control protein. Untransfected DK1 cells showed no cross reactivity with the anti-Tag antibody, whereas transfected cells, DKN, cross-reacted with the anti-Tag antibody.

and CCM2.5. DKN1 cells cultured in CCM2.5 exhibited a two-fold increase in overall growth by day 10 (94.3×10^3 for CCM9 versus 195.5×10^3 for CCM2.5) compared with cells cultured in CCM containing 9 mM L-glutamine. The 2.5 mM concentration of L-glutamine was maintained for the remainder of the study.

Cells were then treated with two known antioxidants, reduced glutathione (GSH) and *N*-acetylcysteine (NAC). The addition of 2.0 mM GSH to DKN1 cells grown in CCM9 restored overall growth to the approximate level of cells grown in CCM2.5 without GSH (Fig. 3, panel B). By day 10 the overall numbers of cells grown in CCM9 plus 2.0 mM GSH were slightly higher than cells grown in CCM2.5 (265.6×10^3 versus 195.5×10^3). By day 12 they had increased to 2.5 fold higher and by day 14 cells grown in CCM9 plus 2.0 mM GSH showed a growth level 4.4 times greater (panel B) than did cells grown in CCM9 alone (panel A). This increased rate of growth was statistically significant (*P* < 0.05) for days 10–14.

DKN1 cells grown in CCM2.5 supplemented with GSH also showed a dramatic difference in rate of growth when compared to cells grown in CCM2.5. In-

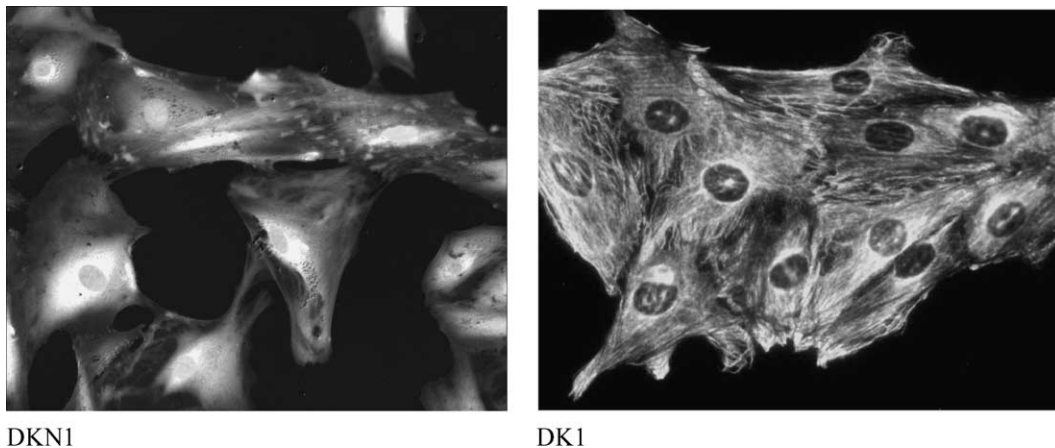


Fig. 2. Detection of intracellular cytokeratin in transfected DK1 cells. DKN1 and DK1 cells were evaluated for the presence of cytokeratin using immunofluorescence methods. Cells were grown in CCM2.5 medium and evaluated for the presence of cytokeratin using mouse anti-cytokeratin, mouse anti- α smooth muscle actin, mouse anti-desmin, or mouse anti-vimentin, and goat anti-mouse IgG from Sigma, St. Louis, MO. Cultures were blocked with 5% normal goat serum, incubated overnight with primary antibody, and detected with fluorescein-conjugated goat anti-mouse secondary antibody. Slides were overlaid with a coverglass and Prolong antifade mounting reagent (Molecular Probes, Eugene, OR), and evaluated using fluorescence microscopy.

terestingly, there was a statistically significant ($P < 0.05$) decrease in proliferation when cells were grown in the lower concentration of L-glutamine (CCM2.5) plus GSH (Fig. 3, panel B).

DKN1 cells grown in CCM9 plus NAC, compared to cells grown in CCM9 with no NAC supplementation, showed a statistically significant decrease ($P < 0.05$) by day 12 (Fig. 3, panel C). Cells grown in CCM2.5 supplemented with NAC also exhibited a significant decrease ($P < 0.05$) for days 10 and 12 as compared to those grown in CCM2.5 alone.

4. Discussion

Practical in vitro methods are essential for preliminary toxicological studies in cetaceans, virtually all of which are protected species. The development and characterization of a continuous bottlenose dolphin cell line would be particularly advantageous since governmental regulations limit the access of most investigators to live animals or fresh cetacean tissues. In this study we have developed a bottlenose dolphin cell line that is epithelial in origin, as evidenced by the presence of intracellular cytokeratin. This cell line, DKN1, is also apparently immortalized due to incorporation

of the DNA sequence for SV40 large tumor antigen (Tag), and has been continuously cultured for 8 years. We have demonstrated that DKN1 cells do not require high levels of L-glutamine during culture as previously believed for cetacean cells.

Cetacean cells have historically been difficult to grow in culture (Andrews et al., 1973; Smith et al., 1987). Successfully cultured lines such as Sp1K and BWK require an enriched medium which contains three times the standard concentrations of vitamins and amino acids (Nielson et al., 1986; Hay et al., 1988). Because of these factors, the original line established in this laboratory was maintained in an enriched medium which included a 9.0 mM concentration of L-glutamine (Carvan et al., 1994). Eukaryotic cells are reported to metabolize glutamine to provide 30–65% of the energy requirements for growth (Reitzer et al., 1979; Zielke et al., 1984). Because many cell lines require exogenous glutamine supplementation, it has been considered a mandatory component of culture media for cetacean cells, with final concentrations ranging between 2.0 and 5.0 mM (Janicki and Goldstein, 1969). More recent studies have shown that some cells can be maintained in glutamine-free medium with no adverse effects on growth (Bolon et al., 1995). In addition glutamine metabolism

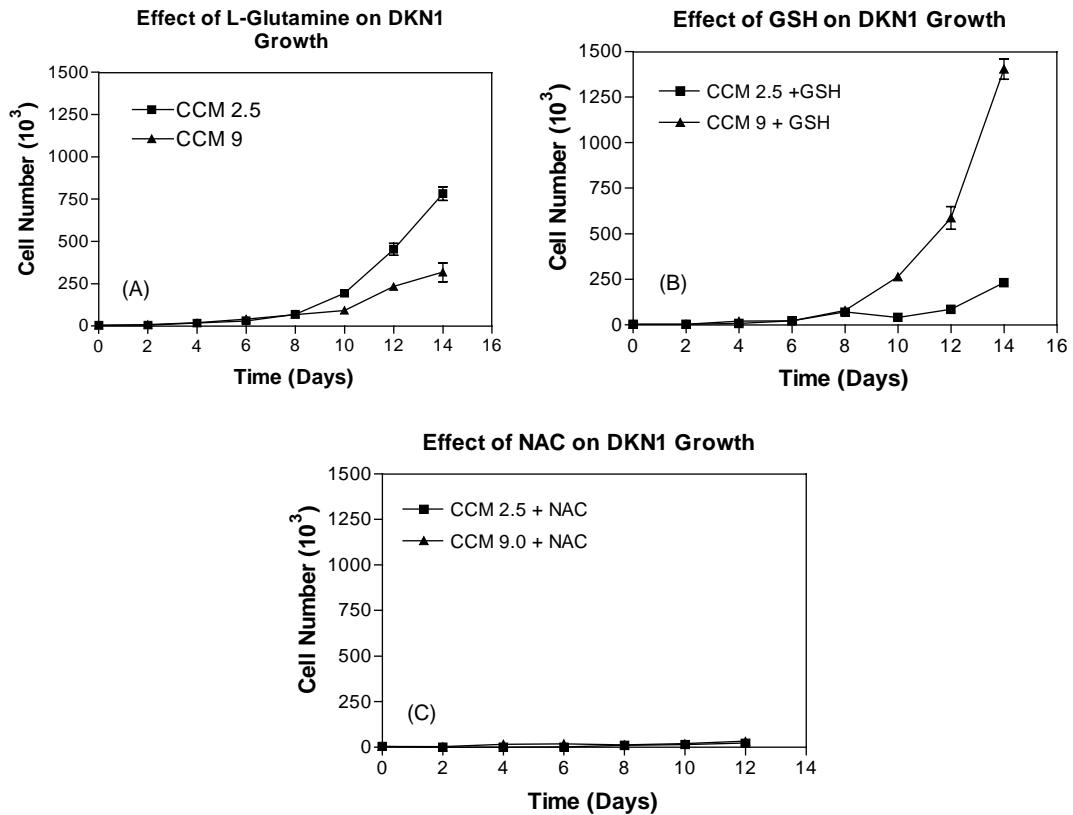


Fig. 3. Effects of L-glutamine, glutathione, and *N*-acetylcysteine on DKN1 growth. Panel A compares the growth rates between cells cultured in the original CCM which contained three times the standard L-glutamine concentration (9.0 mM) and a modified CCM containing 2.5 mM L-glutamine. By day 10 cell numbers were two times higher for cells grown in the lower concentration of L-glutamine. Panel B is a comparison of growth rates after the addition of 2.0 mM reduced glutathione to each culture medium. GSH addition improved the growth rate for cells cultured in 9.0 mM L-glutamine ($P < 0.05$ days 10–14). However, the addition of GSH to cells cultured in CCM 2.5 exhibited a statistically significant decrease in overall cell numbers ($P < 0.05$ days 10–14). Panel C demonstrates the effect of 2.0 mM *N*-acetylcysteine on cell growth. Growth was severely suppressed for the transfected cells grown in both concentrations of L-glutamine ($P < 0.05$ days 8–12).

creates major problems for certain cell types because of the ammonia generated as a metabolic waste product. When cultured in CCM containing L-glutamine at a level only 27.8% as high as has previously been considered an optimal range, DKN1 cells showed a significant growth advantage, approximately 2-fold as early as day 10 (Fig. 3), compared with cells grown in the higher L-glutamine concentrations.

For many cell lines the addition of antioxidants increases the survivability of the cells under adverse conditions. Reduced glutathione and its synthetic precursor *N*-acetylcysteine are routinely used in cell culture as antioxidants. Studies with different cell

types typically show these compounds to be protective against ROS induced injury. Both GSH (2.0 mM) and *N*-acetylcysteine (1.0 mM), when added individually to the culture medium of porcine primordial germ cells (PGC), improved the survival rate in vitro (Lee et al., 2000). Therefore, we decided to culture the transfected cell line in these two routinely utilized antioxidants. However, when either GSH or NAC were added to the medium in this study, the only cells which benefited were DKN1 cells grown in CCM9. For all other treatment groups the GSH and the NAC inhibited cell proliferation. Growth rates were so suppressed in NAC supplemented media that the

study was discontinued after 12 days instead of the normal 14.

While growth suppression in DKN1 cells after the addition of antioxidants appears to be counterintuitive, the explanation may be related to the concentrations used in this study (2.0 mM). Many investigators add 1.0–2.0 mM concentrations of GSH or NAC, however if the cells are able to adequately synthesize GSH, additional supplementation may not be necessary and may actually be detrimental. Two major potential explanations exist to explain the results in this study.

The first possible explanation of the decreased requirement for GSH is that addition of excess GSH may cause a disruption of the reduced glutathione to oxidized glutathione ratio, GSH:GSSG, with possible induced changes in intracellular hydrogen peroxide levels due to the thiols themselves. Imbalances of the thiol-disulfide ratio are known to irreversibly modify proteins, including both enzymes and structural proteins, leading to aberrant cell function (Gilbert, 1990). The oxidation of sulfhydryl groups can result in protein disulfides or protein S-thiolation, and disulfide bonds are acutely important during protein folding. Cellular proteins which become S-thiolated are unstable and denature at 37 °C instead of the more typical 60 °C (Freeman et al., 1995), and concentrations of GSH and NAC above 10.0 mM in the culture media are reported to cause dose-dependent decreases in cellular protein levels in rat pheochromocytoma cells (Offen et al., 1996).

The second possible explanation is related to the interaction of thiols with culture medium components as reported by Long and Halliwell (2001). Both GSH and NAC were shown to generate high levels of H₂O₂, up to 15 and 19 μM, respectively, when allowed to incubate in DMEM. High levels of hydrogen peroxide are known to initiate apoptosis and/or necrosis in cultured cells.

These results demonstrate the impact cell culture medium components can have on experimental data. Parameters for cell culture need to be clearly defined through preliminary experiments on the cell line of interest because changing one component can have a direct impact on future experiments and the eventual interpretation of results. This research not only presents a new transformed cell line for use in marine mammal research, it leaves open the avenue for further exploration of glutamine metabolism in cetacean cells and

questions the requirement of cetacean cells for exogenous antioxidant compounds.

Acknowledgements

This work was supported in part by USEPA award 430891, NOAA Sea Grant award 404227, an NIEHS award to the Center for Environmental and Rural Health R823209, an NIEHS toxicology training grant P30 ES09106, and a NIOSH award U50 07541.

References

- Aguilar, A., Borrell, A., 1991. Abnormal PCB levels in striped dolphins affected by the western Mediterranean epizootic. In: Proceedings of the Ninth Biennial Conference on the Biology of Marine Mammals (Symposium on Marine Mammals as Biological Indicators). Abstract 1115.
- Almeida, M.G., Fanini, F., Davino, S.C., Aznar, A.E., Koch, O.R., Barros, S.B.M., 1997. Pro- and antioxidant parameters in rat liver after short-term exposure to hexachlorobenzene. *Hum. Exp. Toxicol.* 16, 257–261.
- Andrews, J.C., Dill, F.J., Masui, S., Fisher, H.D., 1973. The chromosome complement of the narwhal (*Monodon monoceros*). *Canadian J. Genet Cytol.* 15, 349–353.
- Bielec, P.E., Gallagher, D.S., Womack, J.E., Busbee, D.L., 1998. Homologies between human and dolphin chromosomes detected by heterologous chromosome painting. *Cytogenet. Cell Genet.* 81, 18–26.
- Bielec, P.E., Riggs, P., Womack, J.E., Busbee, D.L., 1997. A high-resolution GBG-banded karyotype of the Atlantic bottlenose dolphin, *Tursiops truncatus*: generation of an idiogram, and NOR localization by fluorescence in situ hybridization. *Cytogenet. Cell Genet.* 78, 6–11.
- Bolon, C., Chauvin, M., Simonnet, H., Gauthier, C., Baverel, G., 1995. Growth of cultured rabbit renal tubular cells does not require exogenous glutamine. *Kidney Int.* 47, 299–305.
- Carvan, M.J., Santostefano, M., Safe, S., Busbee, D., 1994. Characterization of a bottlenose dolphin (*Tursiops truncatus*) kidney epithelial cell line. *Marine Mammal Sci.* 10, 52–69.
- Carvan, M.J., Flood, L.P., Campbell, B.D., Busbee, D.L., 1995. Effects of benzo(a)pyrene on CDK cells: Inhibition of proliferation and initiation of DNA damage. *Chemosphere* 30 (1), 187–198.
- Finklea, B., Miller, G., Busbee, D., 2000. Polychlorinated biphenyl residues in blubber of Atlantic bottlenose dolphins (*Tursiops truncatus*) that stranded and died at Matagorda Bay. *Bull. Environ. Contam. Toxicol.* 64 (3), 323–332.
- Freeman, M.L., Borrelli, M.J., Syed, K., Senisterra, G., Stafford, D.M., Lepock, J.R., 1995. Characterization of a signal generated by oxidation of protein thiols that activates the heat shock transcription factor. *J. Cell. Physiol.* 164, 356–366.
- Gilbert, H.F., 1990. Molecular and cellular aspects of thiol-disulfide exchange. *Adv. Enzymol. Rel. Areas Mol. Biol.* 63, 69–172.

- Hassoun, E.A., Wilt, S.C., DeVito, M.J., Vanbirgelen, A., Alsharif, N.Z., Birnbaum, L.S., Stohs, S.J., 1998. Induction of oxidative stress in brain tissues of mice after subchronic exposure after 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol. Sci.* 42, 23–27.
- Hay, R., Macy, M., Chen, T.R., McClintock, P., Reid, Y., 1988. American Type Culture Collection Catalogue of Cell lines and Hybridomas. ATCC, Rockville, MD.
- Janicki, R.H., Goldstein, L., 1969. Glutamine synthetase and renal ammonia metabolism. *Am. J. Physiol.* 216, 1107–1110.
- Kuehl, D.W., Haebler, R., Potter, C., 1991. Chemical residues in dolphins from the US. Atlantic coast including Atlantic bottlenose obtained during the 1987/88 mass mortality. *Chemosphere* 22, 1071–1084.
- Lee, C., Weeks, R., Johnson, G., Bazer, F., Piedrahita, J., 2000. Effects of protease inhibitors and antioxidants on in vitro survival of porcine primordial germ cells. *Biol. Reprod.* 63, 887–897.
- Long, L., Halliwell, B., 2001. Oxidation and generation of hydrogen peroxide by thiol compounds in commonly used cell culture media. *Biochem. Biophys. Res. Commun.* 286, 991–994.
- Nielson, O., Kelly, R.S., Lillie, W.R., Clayton, J.W., Yoneyama, B.S., Fujioka, R.S., 1986. Characteristics of a cell line from beluga whale (*Delphinapterus leucas*). *In Vitro* 22, 21.
- Offen, D., Ziv, I., Sternin, H., Melamed, E., Hochman, A., 1996. Prevention of dopamine-induced cell death by thiol antioxidants: possible implications for treatment of Parkinson's Disease. *Exp. Neurol.* 141, 32–39.
- Reitzer, L.J., Wice, B.M., Kennell, D., 1979. Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. *J. Biol. Chem.* 254, 2669–2676.
- Scott, M.D., Wells, R.S., Irvine, A.F., 1990. A long term study of bottlenose dolphins on the west coast of Florida. In: Leatherwood, S., Reeves, R.R. (Eds.), *The Bottlenose Dolphin*. Academic Press, San Diego, pp. 235–244.
- Shertzer, H.G., Nebert, D.W., Puga, A., Ary, M., Sonntag, D., Dixon, K., Robinson, L.J., Cianciolo, E., Dalton, T., 1998. Dioxin causes a sustained oxidative stress response in the mouse. *Biochem. Biophys. Res. Commun.* 253, 44–448.
- Smith, A.W., Skilling, D.E., Benirschke, K., Albert, T.F., Barlough, J.E., 1987. Serology and virology of the bowhead whale (*Balaena mysticetus*). *J. Wildlife Dis.* 23, 92–98.
- Srivastava, V., Schroeder, M., Busbee, D., 1993. Activity of DNA polymerase alpha in pSV3.neo plasmid transformed human fibroblasts. *Int. J. Biochem.* 25, 385–395.
- Zielke, H.R., Zielke, C.L., Ozand, P.T., 1984. Glutamine: a major energy source for cultured mammalian cells. *Federation Proc.* 443, 121–125.