Mass Culture and Characterization of Tumor Cells From a Naturally Occurring Invertebrate Cancer Model: Applications for Human and Animal Disease and Environmental Health

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Abstract. On the northeastern coast of the United States and Canada, Mya arenaria, the soft shell clam, develops a diffuse, hemopoetic tumor (a fatal leukemia-like cancer) resulting from inactivation of p53-like family member proteins. These malignant cells provide a model for an unrelated set of human cancer cells that are also characterized by mortalin-based cytoplasmic sequestration of wild-type p53 tumor suppressor protein (mortalin is the mitochondrial Hsp70 protein). Here we describe methods for mass culture and long-term storage of tumor cells from this cancer. These are the first successful efforts at maintaining malignant cells from any marine invertebrate in vitro. Following passage (subculture), these cultures undergo transition from primary cultures to non-immortalized cell lines that continue to proliferate and do not re-differentiate the normal hemocyte phenotype. We also characterize normal clam hemocytes and the pathology of cancerous clam hemocytes in vitro and in vivo using light and electron microscopy, cyto- and immunocytochemistry, molecular biology, and a phagocytosis assay. Our protocols provide biomedical and environmental researchers with ready access to this naturally occurring cancer model. We discuss the clam cancer model regarding (a) human health and disease; (b) animal health, disease, and aquaculture; (c) environmental health monitoring; and (d) future research directions.

Introduction

Soft shell clams display a fatal cancer at multiple New England sites, south to Chesapeake Bay, and north into Canada (individuals from many commercial sources have ≥1% incidence of this disease). The highest incidence of clams with 100% cancerous cells in the hemolymph has been recorded from New Bedford Harbor, Massachusetts (≈15%), which is an Environmental Protection Agency Superfund site characterized by high levels of contaminants identified as mutagenic or carcinogenic in the sediments and water column. The contaminants include polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans, polycyclic aromatic hydrocarbons, and several trace metals in toxic concentrations (Reinisch et al., 1984; Laevitt et al., 1990; Garton et al., 1992; Ford et al., 2005).

It is unclear if clam hemocyte cancer arises from a clonal population of stem cells, and it is also unknown where these malignant cells originate. Cuénot (1891) suggested that bivalve hemocytes normally develop in “lymphatic tissue” where the gills are suspended from the visceral mass. Because the specific tissue of origin for this tumor is unknown, it is not appropriate to classify clam hemocyte cancer as leukemia. This disease has been designated as a systemic disseminated neoplasia by the now defunct Registry of Tumors in Lower Animals (formerly maintained by the...
National Cancer Institute), although the most common descriptors in the literature are hemopoetic, hematopoetic, or hemic neoplasia (Barber, 2004). We will use the phrase cancerous clam hemocytes (CCH) to describe these malignant cells.

In this study, we provide protocols for culturing CCH from a naturally occurring disease in the soft shell clam, *Mya arenaria* Linnaeus, in which highly conserved homologs for human wild-type p53 family proteins (GenBank Accession numbers: p53-like, AF253323; p63/73-like, AF253324) are nonfunctional because they are sequestered in the cytoplasm of CCH (Kelley et al., 2001; Walker et al., 2006; Böttger et al., 2008). This phenomenon in clams reflects that seen in a subset of human cancers, yields a similar phenotype, and results in loss of p53 transcriptional function (Wadhwa et al., 2003; Kaul et al., 2007). In all these cases, wild type p53 does not initiate transcription of downstream genes because it cannot bind DNA. There are multiple mechanisms responsible for cytoplasmic sequestration of p53 in human cancer cells, and most of these mechanisms are poorly understood (Kaul et al., 2000). Soft shell clam hemocyte cancer provides excellent in vivo and in vitro models for human and other animal cancers displaying cytoplasmic sequestration of the p53 tumor suppressor by mortalin, the mitochondrial Hsp70 protein (Kelley et al., 2001; Dundas et al., 2005; Walker et al., 2006). Two variants of mortalin are overexpressed in cancerous clam hemocytes (Böttger et al., 2008). There is a full-length variant (GenBank Accession #AY326398) and a truncated variant (GenBank Accession #EF576660), the latter of which is apparently a splice variant of the former and is missing exon 3 that includes the ATP-binding and ATPase domain. Since ATP binding and hydrolysis are essential for releasing p53 from the mortalin/p53 complexes in mammals, it is possible that the truncated variant is unable to release p53. Many Hsp70 proteins are inducibly expressed in response to environmental stressors and have been termed “central coordinators” of cellular function (Beere, 2001).

The etiology of clam hemocyte cancer is unknown and virtually uninvestigated. Competing hypotheses, based on limited data, suggest either environmental or viral origins for the disease, although other factors such as elevated temperatures may also be involved. Several studies report either significant or no correlation with pollution at different sites, but most of these were conducted after a major contamination or environmental event and do not benefit from multiple samplings over time (Walker et al., 1981; Appeldoorn et al., 1984; Laevitt et al., 1990; Garton et al., 1992). Independent investigations supporting a viral origin demonstrate that the disease can be transplanted to normal clams by inoculation of hemocytes or cell-free hemolymph from diseased clams (McLaughlin et al., 1992; Sunila, 1992; Wineberg et al., 1997; Renault and Novoa, 2004). None of these studies has unequivocally identified a causal virus, and detection of viral particles has been hampered by the absence of cell lines from the soft shell clam (other than a heart cell line; Kleinschuster et al., 1996) for viral isolation and characterization (Brown, 1980; Oprandy and Chang, 1983; House et al., 1998; Renault and Novoa, 2004).

Determining relationships between the molecular biology of clam hemocyte cancer and contaminant, viral, or other mechanisms that might induce this disease have been hampered by the lack of an experimentally accessible version of the malignancy. Here we describe methods for mass culture and long-term storage of CCH both in vitro and in liquid nitrogen; we also characterize normal clam hemocytes (NCH) in vivo and the pathology of CCH in vitro and in vivo. Following passage (subculture), CCH successfully transition from primary cultures to non-immortalized cancer cell lines that continue to proliferate and do not revert to the normal clam hemocyte phenotype (as defined for human cells in suspension culture by Schaeffer, 1984; Masters, 2000). We also confirm and extend observations that normal clams will develop cancer after inoculation with different CCH/hemolymph combinations (augmented with clam culture medium), providing a transplantable tumor model for amplifying CCH in vivo (Ostrand-Rosenberg, 2004). We hope that the availability of in vitro and in vivo versions of this naturally occurring disease will provide biomedical and environmental researchers with ready access to this cancer model and promote studies on the interactions of genes and environmental factors that might be involved in generating this disease in soft shell clams and similar mortalin-based diseases in other bivalve molluscs and in humans.

### Materials and Methods

#### Experimental clams

Soft shell clams (*n* = 70–150) were collected at the lowest tides of each month from sand flats on Marsh Island in New Bedford Harbor at Fairhaven, Massachusetts (41°38.0′N 70°55.0′ N) and were maintained at the University of New Hampshire Coastal Marine Laboratory, New Castle, New Hampshire. For biopsy, a small aliquot (10 µl) of hemolymph was aspirated, using a 26-gauge needle, from the pericardial sinus and incubated in a 96-well microtiter plate for 2 h at 8 °C. Clam hemolymph was classified as normal (100% attached motile NCH and 0% round, non-motile CCH), early incipient cancerous (1%–50% CCH), late incipient cancerous (50%–99% CCH), or fully cancerous (100% CCH). Over an 8-year period in 61 collections, the average number of clams with hemolymph that contained 100% CCH was 3.99% ± 0.381% (SD); the range was 0%–15.2% (unpubl. data).
Light and electron microscopy and cyto- and immunocytochemistry of clam hemocytes

Cytospins (Shandon Cytospin 3) of freshly collected hemocytes (± 50–100 μl) from individuals with hemolymph that was normal (0% CCH), fully cancerous (100% CCH), or from CCH maintained in vitro were treated with the following stains and enzyme assays normally used to characterize human blood cells: Romanovsky stain (Thermo-Shandon, Kwik Diff stain kit), periodic acid–Schiff (PAS, Sigma 395-B), Sudan black B (SBB, Sigma 380-B), Oil red O (Humason, 1979), myeloperoxidase (MPO, Sigma 391-A), acid phosphatase (Sigma 181-A), alkaline phosphatase (Sigma 095-B), and naphthol AS-D chloroacetate (specific esterase (Sigma 90-C2) and α-naphthol acetate (non-specific esterase (Sigma 90-A1). Negative controls (no substrate) were conducted for all assays.

Cytospins of ±75 μl of freshly collected hemocytes from clams with hemolymph that was normal (0% CCH) and fully cancerous (100% CCH) or from CCH maintained in vitro were fixed and permeabilized by immersion in acetone. Primary antibodies (1:50 μl primary antibody) for immunohistochemistry were clam p53 and p63/73 (rabbit anti-clam polyclonal to identify members of the clam p53 family of proteins; Kelley et al., 2001), clam mortalin (rabbit anti-clam polyclonal; Walker et al., 2006), α-tubulin (clone DM-1A, mouse anti-chicken monoclonal, ICN; to demonstrate the microtubular array involved in the transport of clam p53 family proteins), and clam cancerous hemocyte-specific membrane protein (1E10, mouse anti-clam monoclonal; Miosky et al., 1989; Stephens et al., 2001). Resulting preparations were developed with the appropriate peroxidase-labeled secondary antibody (Vectastain ABC Elite IgG kit, Vector Laboratories) and diaminobenzidine (DAB, Vector Laboratories). To localize variants of clam p53 or mortalin family members, polyclonal antibodies (DAB, Vector Laboratories) and diaminobenzidine (DAB, Vector Laboratories) were directly conjugated to QDot antibody conjugation kits (Invitrogen Corp., CA) (Walker 655 (red fluorescence, p53) and 525 (green fluorescence, p53 or mortalin family members, polyclonal antibodies were assessed.

Cancerous clam hemocytes inoculated into normal clams

Adult soft shelled clams were purchased from a local seafood market, maintained at the University of New Hampshire Coastal Marine Laboratory for 1 week prior to treatment, and biopsied to assess disease status (only 0% CCH were used). Clams (n = 20) were divided into five groups and injected with 200 μl of treatment-dependent fluid. Control individuals (A) received 200 μl of clam culture medium only, without addition of fetal bovine serum, antibiotics, or pH indicators (Table 1).

Treatment regimes for normal clams with CCH, hemolymph, or both were prepared as follows to obtain a final hemocyte count of 2.5 × 10^6 cells/ml. (B) CCH in diseased clam hemolymph: 46 μl of diseased clam hemolymph was combined with 154 μl of clam culture medium for each inoculation. (C) CCH in clam culture medium: CCH were isolated by centrifugation (60 RCF for 10 min) and resuspended in clam culture medium. (D) Diseased clam hemolymph: 46 μl of diseased clam hemolymph was centrifuged at 60 RCF for 10 min, after which the supernatant was collected and the pelleted CCH discarded. (E) Lysed CCH in diseased clam hemolymph: hemolymph was combined with clam culture medium as in treatment B, and CCH were lysed by centrifugation at 10,701 RCF for 15 min (lysis was confirmed by microscopy).

After inoculation with treatments A–E, treated clams were sampled each week for 3 months by withdrawing 10
μl of hemolymph from the pericardial sinus, dispensing it into 96-well flat-bottom plates, incubating it at 8 °C for 2 h, and scoring it on a Zeiss IM microscope (Carl Zeiss, Inc., Thornwood, NJ).

**Analysis of genomic DNA from normal clam hemocytes in vivo and cancerous clam hemocytes in vivo and in vitro**

To confirm that CCH develop from normal clam hemocytes and are not of foreign origin (Mackin and Schlicht, 1976), total genomic DNA was extracted from freshly collected soft shell clam hemocytes from normal (0% CCH) and fully cancerous (100% CCH) clam hemolymph or from 100% CCH in vitro, using a Wizard Genomic DNA purification kit (Promega, Madison, WI). Double-stranded amplifications were performed using a forward primer 16sar (5'-CGCCTGTTTATCAAACAT-3') that corresponded to the 16S mitochondrial rDNA gene consensus primer. The reverse primer 16sbr (5'-CCGGTTGA ACTCAGAT-CATGT-3') was designed to position 12887 of the **Drosophila** mitochondrial genome (Clary and Wolstenholme, 1985). Double-stranded products were amplified and used as templates in cycle sequencing reactions (Applied Biosystems Inc., Foster City, CA). For each sample, both strands of amplified DNA were sequenced and results deposited in GenBank under accession numbers AF149119 (NCH in vivo), AF149120 (CCH in vivo), and AF149121 (CCH in vitro). Sequence editing and alignment were done using the SeqEd program (ver. 1.0.3, ABI).

**Phagocytosis assay for hemocytes**

Hemocytes from freshly collected normal (0% CCH) and fully cancerous (100% CCH) clam hemolymph and from CCH in vitro were washed (3 times) with filtered natural seawater (FSW, 0.45 μm), seeded in FSW at 1.5–2.0 × 10^5 cells ml^{-1}, and allowed to adhere overnight at 8 °C. The disparity in numbers of hemocytes present in the hemolymph from single normal clams (1–6 × 10^6 cells ml^{-1}) and single clams with hemocyte cancer (5 × 10^8 hemocytes ml^{-1}) required pooling the hemolymph from several normal clams (n = 3–5 clams per replicate run). Cultures were brought to a final volume of 3 ml FSW containing 1.0-μm-diameter fluorescent microspheres at an approximate density of 25 per hemocyte (excitation 490 nm, emission maximum 515 nm; Fluospheres, Molecular Probes, Inc.).

**Table 1**

*Culture medium for maintaining cancerous clam hemocytes: ingredients listed were added to 9.3 g Eagle’s MEM (Sigma, M-4144) to make 2 l at final osmolarity of 1100-1150 mOsm, modified from Sible et al. (1992)*

<table>
<thead>
<tr>
<th>Additional ingredients</th>
<th>Grams</th>
<th>Amount added</th>
<th>Absolute final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SALTS (added in order)</strong></td>
<td></td>
<td></td>
<td>mmol l^{-1}</td>
</tr>
<tr>
<td>CaCl_2</td>
<td>3.647 g</td>
<td>17.6</td>
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<tr>
<td>KCl</td>
<td>1.358 g</td>
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</tr>
<tr>
<td>MgCl_2 × 6H_2O</td>
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<tr>
<td>MgSO_4 × 7H_2O</td>
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<td>12.91</td>
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<tr>
<td>NaCl</td>
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<tr>
<td>NaHCO_3</td>
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<td>11.90</td>
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</tr>
<tr>
<td>Heps Buffer</td>
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<tr>
<td>Glucose</td>
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<tr>
<td>Glutamine</td>
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<td><strong>AMINO ACIDS</strong></td>
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<td>l-Alanine</td>
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<tr>
<td>l-Asparagine</td>
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<td>l-Aspartic acid</td>
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<td>Proline</td>
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<tr>
<td>Glycine</td>
<td>1.0 ml from stock (0.188 g/25 ml) for 0.05 ml</td>
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<td><strong>ANTIBIOTICS</strong></td>
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<td></td>
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<tr>
<td>Ampicillin</td>
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</tr>
<tr>
<td>Streptomycin</td>
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<tr>
<td>Penicillin</td>
<td>200</td>
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<tr>
<td><strong>SERUM %</strong></td>
<td></td>
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<td></td>
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<tr>
<td>FBS (heat inactivated)</td>
<td></td>
<td>10</td>
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Assays \((n = 3)\) were incubated for 24 h at 8 °C, and the percentage of hemocytes \((n = 100)\) containing microspheres was calculated and scored as follows: 1+ contained 1–2 microspheres, 2+ contained 3–4 microspheres, 3+ contained 5–8 microspheres, and 4+ contained microspheres too numerous to count. Data were analyzed using a nonparametric Kruskall-Wallis test for multiple groups (Sokal and Rohlf, 1981).

**Results**

**Cyto- and immunochemistry of normal clam hemocytes (NCH) in vivo**

Normal clam hemolymph contains terminally differentiated hemocytes (Seiler and Morse, 1988) at an average concentration of \(\approx 1–6 \times 10^6\) cells ml\(^{-1}\). Both agranular and granular hemocytes are present and actively mobile; both also adhere tightly to plastic and glass and can be lost in processing. NCH have an average diameter of 7–9 \(\mu\)m, have a mean nuclear-to-cytoplasmic volume ratio of 1:10, and are phagocytic (Beckmann et al., 1992) (Figs. 1a–c; 2a–c). Results of cyto- and immunochemistry for NCH from normal clams (0% CCH) are shown in Table 2; comparative literature values indicate considerable variation for several of these stains and enzyme assays (Huffman and Tripp, 1982; Beckmann et al., 1992). Strong positive reactions were observed for PAS, Oil red O (5–15 vesicles cell\(^{-1}\); Fig. 2f), \(\alpha\)-tubulin (Fig. 2g), nonspecific esterase, variants of clam p53 family proteins (Fig. 2h), and for both variants of mortalin in the cytoplasm (Fig. 2i) and for 1E10 at the cell membrane. Weak (<20%) to negative reactions resulted for all other cyto- and immunoocytochemical tests performed (Sudan black B, myeloperoxidase, acid and alkaline phosphatases, and specific esterase). Clam p53 and mortalin are often concentrated near the centriole or at the nuclear membrane and otherwise mirror those of the well-developed microtubular array (Fig. 2g, h).

**Cancerous clam hemocytes in vitro before and after cryopreservation and after inoculation of normal clams CCH in vitro**

A growth curve for CCH in vitro was established using daily hemocyte counts from seeding (= time zero) until senescence (\(\approx 80\) h without passage). Including an initial lag phase, the intrinsic growth rate \((K_i)\), calculated from a regression of log growth rates with time, was \(0.012\ h^{-1}\) (ANOVA: \(P < 0.01; r^2 = 0.987\)) at 8 °C. Doubling time \((k = 0.69/K_i)\) was calculated at 2.4 d\(^{-1}\) or 0.42 divisions d\(^{-1}\) (Fig. 3a). A growth curve was also established for cryopreserved CCH revived from freezing (Fig. 3b). These cells also showed an initial lag phase with a growth constant of \(K_i = 0.016\ h^{-1}\) (ANOVA: \(P < 0.01; r^2 > 0.9\)) and a doubling time averaging \(1.8\ d^{-1}\) or 0.56 divisions d\(^{-1}\). After passing these cells into new clam culture medium, the CCH from either of these treatments were maintained in suspension by repeated passages as non-immortalized cell lines for 3 months (data not shown). Longer maintenance was not attempted.

**Cyto- and immunochemistry of CCH in vitro**

CCH in vitro are nearly round, average 5–10 \(\mu\)m in diameter, have a nuclear-to-cytoplasmic volume ratio of 1:1, attach weakly to plastic and glass by short pseudopodia, and display obvious mitotic figures (Fig. 4a,b). Results of other cyto- and immunoocytochemical tests of CCH in vitro (Table 2) include strong positive reactions for PAS, Oil red O (5–15 vesicles cell\(^{-1}\), \(\alpha\)-tubulin, nonspecific esterase, all variants of clam p53 and mortalin proteins in the nucleus, and for both variants of p53 and mortalin genes were not distinguished from each other with our polyclonal antibodies). Weak (<10%) to negative reactions resulted for all other cyto- and immunocytochemical tests performed (Sudan black B, myeloperoxidase, acid and alkaline phosphatases, specific and nonspecific esterases, and 1E10).

**Cyto- and immunochemistry of cancerous clam hemocytes (CCH) in vivo**

Clams used in this study contained 95%–100% CCH \((5 \times 10^8\) cells ml\(^{-1}\)) with an average diameter of 7–10 \(\mu\)m and a mean nuclear-to-cytoplasmic volume ratio of 1:1 (Figs. 1d–f; 2d–i). These nearly round, mitotically active hemocytes have a monotonous appearance, are often polyploid (Siah et al., 2008), attach only loosely to plastic or glass by short pseudopodia, and are not motile or phagocytic (Beckmann et al., 1992). Results for cyto- and immunochemistry of freshly collected CCH are shown in Table 2 and Fig. 2f–i. Comparative literature values indicate considerable variation for several of these stains and enzyme assays in clams collected in different seasons and at different collection sites (Huffman and Tripp, 1982; Beckmann et al., 1992). Strong positive reactions were observed for PAS, Oil red O (5–15 vesicles cell\(^{-1}\); Fig. 2f), \(\alpha\)-tubulin (Fig. 2g), nonspecific esterase, variants of clam p53 family proteins (Fig. 2h), and for both variants of mortalin in the cytoplasm (Fig. 2i) and for 1E10 at the cell membrane. Weak (<20%) to negative reactions resulted for all other cyto- and immunoocytochemical tests performed (Sudan black B, myeloperoxidase, acid and alkaline phosphatases, and specific esterase). Clam p53 and mortalin are often concentrated near the centriole or at the nuclear membrane and otherwise mirror those of the well-developed microtubular array (Fig. 2g, h).
Figure 1. Light and electron microscopy of normal clam hemocytes (NCH; a–c) and cancerous clam hemocytes (CCH; d–f) in vivo. (a) Longitudinal section through the gill of a normal soft shell clam; arrow indicates hemolymph sinus; scale bar = 100 μm. (b) Higher magnification view to show both agranulocytic and granulocytic (arrow) hemocytes in the hemolymph sinus; scale bar = 50 μm. (c) Cross section through the mature testis of a normal soft shell clam, showing clusters of nutritive cells (fc) surrounding darker spermatogenic cells within follicles; hemolymph sinuses penetrate between follicles; scale bar = 100 μm. (d) Longitudinal section through the gill of a diseased soft shell clam, showing CCH in the hemolymph sinus (arrow); scale bar = 100 μm. (e) Higher magnification view showing a CCH in metaphase within the hemolymph sinus (arrow); scale bar = 50 μm. (f) Cross section through the testis of a diseased soft shell clam, showing that the hemolymph sinus contains numerous CCH and fills the space normally occupied by nutritive cells; scale bar = 100 μm. fc, nutritive follicle cells; wt, water tube.
Figure 2. Cyto- and immunocytochemistry of normal clam hemocytes (NCH; a–c) and cancerous clam hemocytes (CCH; d–i) in vivo. (a) Nomarski image of living, freshly collected NCH showing both agranulocytic and granulocytic (arrow) hemocytes. Inset shows cytospin of NCH treated with the polyclonal antibody to clam p53 family members and demonstrates nuclear localization; scale bar \( = 10 \, \mu m \). (b) Transmission electron micrograph of a single granulocytic NCH; scale bar = 5 \( \mu m \). (c) Low-magnification transmission electron micrograph of several agranulocytic NCH; scale bar = 10 \( \mu m \). (d) Nomarski image of living, freshly collected CCH. (e) Plastic section of several CCH showing monotonous appearance of these cells, stained with bromphenol blue. (f) Cytospin of CCH stained with Oil red O to show numerous cytoplasmic vesicles (counterstained with methyl green). (g) Cytospin of several CCH showing localization of Qdot-labeled \( \alpha \)-tubulin in the microtubular array toward one side of the nucleus; n = nucleus; scale bar = 10 \( \mu m \). (h) Cytospin of several CCH showing cytoplasmic localization of p53 family member proteins. (i) Cytospin of several CCH showing cytoplasmic distribution of both splice variants of mortalin. n, nucleus of CCH, Scale bars for d, e, f, h, and i = 20 \( \mu m \).
AF149121 for CCH in vitro, AF149119 for NCH in vivo, and AF149120 for CCH in vivo.

CCH in vivo. With the exception of control individuals that were treated with clam culture medium only and were not exposed to CCH or hemolymph from diseased clams, individuals from all treatments contained CCH 1 week after treatment (Fig. 5). Inoculation with lysed CCH in diseased clam hemolymph (E) resulted in the fastest transmission of clam hemocyte cancer (6.25% of treated clams 3 weeks after injection), while inoculation with intact CCH in hemolymph from diseased clams (B) resulted in fewer CCH (0% of treated clams had 76%–100% CCH) after 3 weeks. Nine weeks after treatment, individuals inoculated with lysed CCH in diseased clam hemolymph (E) had the highest incidence of hemocyte cancer (87.5% of the treated clams had 76%–100% CCH), while clams inoculated with CCH resuspended in clam culture medium (C; Table 1) or with intact CCH in diseased clam hemolymph (B) had a lower incidence of clam hemocyte cancer (24.5% and 5.25% of treated clams had 76%–100% CCH, respectively, Fig. 5). Inoculation with diseased clam hemolymph only (D) resulted in the slowest transmission of clam hemocyte cancer.

Phagocytosis

There was a significant treatment effect of hemocyte type on phagocytic activity (Kruskall-Wallis analysis; \( H \) corrected for ties; 106.61: \( P < 0.001 \)) (Fig. 6). A significantly greater number of NCH phagocytized fluorescent microspheres (65%) compared to either freshly isolated CCH (4%) or CCH in vitro (14%). This significant treatment effect was analyzed by pair-wise multiple comparisons using nonparametric Mann-Whitney \( U \) tests with a corrected experimental-wide \( P \) value of 0.0175. All treatment groups were significantly different at this level of probability using multiple comparisons.

Discussion

In the following sections, we discuss our methods for culture of cancerous clam hemocytes (CCH) and applications for the clam cancer model regarding (a) human health and disease; (b) animal health, disease, and aquaculture; (c) environmental health monitoring; and (d) future research directions.

Methods for in vitro culture and cryopreservation of cancerous clam hemocytes

Culture of CCH was accomplished using a medium and conditions previously employed for gametogenic cells from sea stars (Sible et al., 1992) and modified on the basis of amino acid analysis and atomic absorption spectrometry of clam hemolymph. These are primary cultures originating from clams with hemolymph containing 100% CCH. External, such clams appear normal, but they are lethargic, withdraw slowly into the shell, have flaccid siphons, and eventually die. Also, clams in advanced stages of the disease lose weight and experience negative impacts on gametogenesis (Potts, 1993; Butler et al., 2004).

Immortalized cell lines do not yet exist for any invertebrate tumor, although our ability to passage CCH and maintain cells in vitro for several weeks satisfies all the requirements for transition from a primary culture to a non-immortalized cell line recognized for vertebrate cells in suspension culture (terminology derived from the Society of In Vitro Biology; Schaeffer, 1984). Human cells in suspension that are derived from primary cultures are given this classification after they are first split into new culture ves-
sels and when they subsequently develop a relatively uniform population that proliferates but does not revert structurally to the tissue of origin (Masters, 2000).

An essential factor for successful culture of this kind is the maintenance of CCH at or below the maximum ambient environmental temperature of collection experienced in the winter ($\leq 15 ^\circ C$; $8 ^\circ C$ is optimum). The health of the culture also depends upon the source of CCH. When held in running seawater, clams with 100% CCH die within days, while clams with 0% CCH survive for weeks to months. Diseased clams with low levels of CCH ($<50\%$) will survive for days to weeks. CCH retrieved from diseased clams in the winter are fairly easy to culture and will survive through multiple passages (weeks longer that the source clam), while those from clams collected in summer often yield cultures that die quickly, as does the source clam. In later stages of the cancer, the hemolymph of diseased clams contains many times more CCH than the normal numbers of

Figure 3. Cancerous clam hemocytes (CCH) in vitro. CCH cultured in vitro to evaluate (a) doubling time and (b) growth curve for cryopreserved CCH grown in culture at 8 °C (regression analyses for both flasks were significant—ANOVA; $P < 0.05$). The growth constant is $K_c = 0.016$ for each flask, 0.55 divisions d$^{-1}$ or a doubling time of 1.8 d or 0.55 $^{-1}$; initially, CCH decrease in numbers, then cells enter a log phase and finally decrease again in numbers, unless provided with new culture medium.
hemocytes, has an elevated bacterial load, and is hypoxic owing to the resulting increase in oxygen demand (Sunila, 1991). This latter observation is consistent with the lower phagocytic activity of CCH recorded in this study and by others (Beckmann et al., 1992) (Fig. 6). When grown in our culture medium, which includes antibiotics, hypoxia resulting from bacterial growth is avoided; therefore some other property of CCH must cause a reduction in phagocytosis. Perhaps the poorly developed actin cytoskeleton found in CCH impairs the ability of the cells to phagocytose particles (Beckmann et al., 1992).

Methods for in vivo culture of cancerous clam hemocytes

Hemocyte cancer in clams is an example of a transplantable tumor that is not hampered by two of the characteristics that limit the applicability of mouse transplantable tumor models (Ostrand-Rosenberg, 2004). For example, histocompatibility is not an issue since clams have only innate immunity (Anderson, 1993; Nappi and Ottaviani, 2000; Blaise et al., 2002; Medzhitov and Biron, 2003; Wootton et al., 2003), and injection is not subcutaneous as it is in mouse models, but occurs in an anatomically appropriate site for the tumor (the clam circulatory system). Clam hemocyte cancer is most effectively transferred to normal clams after inoculation of normal clams with lysed CCH in diseased clam hemolymph or intact CCH in clam culture medium (Table 1; Fig 5). Induction of cancer by inoculation of isolated intact CCH or hemolymph from diseased clams is also possible, though the transition occurs much more slowly. Previous studies suggested that hemolymph is essential to provide proteins and mitotic factors for successful transplantation (Sunila, 1992). However, in the present study, cancer was also initiated when CCH were resuspended in clam culture medium where growth and mitotic

Figure 4. Cyto- and immunocytochemistry of cancerous clam hemocytes (CCH) in vitro. (a) Nomarski image of living CCH at 70+h in culture. (b) Romanovski-stained CCH at 70+h culture (arrow indicates mitotic CCH). (c) CCH at 70+h culture treated with cancerous clam hemocyte-specific monoclonal antibody, 1E10, showing localization at the cell surface; inset shows control CCH minus primary 1E10 antibody. Scale bars = 20 μm.
factors were not present. Cancer in the host clam resulting from inoculation of intact CCH undoubtedly depends on their increase in numbers by mitosis within normal clam hemolymph under favorable physiological conditions and results as CCH eventually overgrow host NCH populations that are terminally differentiated and do not divide (Seiler and Morse, 1988). Since all individuals that were inoculated with hemolymph without intact CCH acquired the disease, propagation of clam hemocyte cancer by an unknown virus seems highly likely (Oprandy and Chang, 1983; House et al., 1998; Renault and Novoa, 2004).

Applications for the clam cancer model

(a) Human Health and Disease

Structure and function of clam homologs for Human p53 and p63/73. According to Jessani et al. (2005) the development of cell and animal models that accurately depict human tumorigenesis remains a major goal of cancer research. Analysis of the effects of genotoxic and non-genotoxic compounds on malignancy is currently limited to vertebrate cancer models where tumors are induced (e.g., the mouse model). Additionally, use of vertebrates is subject to strict regulations under National Institutes of Health guidelines. The clam cancer model is more similar to an out-breeding, human clinical population than are those generated from inbred mouse strains intentionally exposed to known tumor viruses. Fly and worm models for human cancers involving mortalin do not exist, principally because adult somatic cells in these organisms do not divide, so an in vivo version of the model is unavailable (Walker and Böttger, 2008). Also, existing fly and worm models have highly derived homologs for p53 only (p63 and p73 are lacking, and the p53 transcriptional activation domain is <2% and 33% conserved, respectively, with that of humans) (Brodsky et al., 2000; Jin et al., 2000; Derry et al., 2001; Schumacher et al., 2001). Homologs for both human p53 and p63/73-like genes have been cloned, and considerable information on the function of these p53-like family proteins exists for soft shell clams (Barker et al., 1997; Van Beneden et al., 1997; Kelley et al., 2001; Stephens et al., 2001; Walker et al., 2006; Böttger et al., 2008; Siah et al., 2008; Walker and Böttger, 2008).

Clam cancer and human colon carcinoma, neuroblastoma, and glioblastoma share similar defects in p53 functionality. Development of non-genotoxic and genotoxic strategies for reactivating p53 function could point out promising cancer

Figure 5. Amplification of cancerous clam hemocytes (CCH) in vivo. Healthy clams (100% NCH) were inoculated in five treatments (200 μl/animal). (A) Clam culture medium —Table 1 (control treatment). (B) CCH in diseased clam hemolymph. (C) CCH in clam culture medium. (D) Diseased clam hemolymph only. (E) Lysed CCH in diseased clam hemolymph. Patterns represent clams with the following percentages of cancer: □ 0% □ 1–25% ■ 26–50% ■ 51–75% ■ 76–100%.
therapies (Martins et al., 2006; Yu, 2006; Fuster et al., 2007; Marx, 2007; Böttger et al., 2008). This is particularly true when the inactivated p53 is wild type. While cytoplasmic sequestration has not yet been recorded for human leukemias or lymphomas, a subset of naturally occurring but unrelated human cancers do retain p53 in the cytoplasm (e.g., undifferentiated neuroblastoma, breast, retinoblastoma, colorectal adenocarcinomas, and glioblastoma (Moll et al., 1992, 1995; Nagpal et al., 2006). In human colorectal cancer cells of some patients and in cancerous clam hemocytes, mortalin is responsible for cytoplasmic sequestration when the latter protein is overexpressed (Dundas et al., 2005; Walker et al., 2006). Dundas et al. (2005) suggest that overexpression of mortalin is also correlated with poor clinical outcome for colorectal cancer and that loss of function of wild-type p53 activity by mortalin binding may also result in colorectal adenocarcinomas. Microarray analyses for a variety of other human cancers have indicated that mortalin is also strongly overexpressed, but experiments demonstrating connections to p53 are unavailable at present.

In the soft shell clam, we have shown that CCH have an intact p53-activating pathway leading to apoptosis and that maintenance of this diffuse tumor requires nuclear absence of p53 dictated by its localization in the cytoplasm (Böttger et al., 2008). Recent studies of CCH used co-immunoprecipitation to demonstrate that clam p53 family proteins and both overexpressed clam variants of human mortalin proteins are complexed together in the cytoplasm of CCH and not in normal clam hemocytes (NCH). This indicates that clam p53 is sequestered in the cytoplasm of CCH by clam mortalin when the latter is overexpressed (Walker et al., 2006). Our subsequent studies have identified both non-genotoxic (using the dye MKT-077; Koya et al., 1996; Wadhwa et al., 2000, 2003; Walker et al., 2006) and genotoxic (using etoposide and mitoxantrone; Böttger et al., 2008) preclinical strategies that might be developed to treat human and other animal cancers in which cytoplasmic sequestration of wild type p53 is mediated by mortalin binding (Dundas et al., 2005). Our recent study of CCH has demonstrated that, as in human diseases (Tomita et al., 2006), both non-genotoxic and genotoxic treatments are effective in inducing apoptosis through transcription when the nucleus of CCH is accessible and also that non-transcription-based induction of apoptosis occurs in CCH through mitochondrial-directed p53 when the nucleus is rendered inaccessible (Walker and Böttger, 2008).

(b) Animal health, disease and aquaculture

Aside from the obvious effects on clam mortality (Barber, 2004), clams in advanced stages of the disease display both loss of tissue weight and impacts on gametogenesis (Potts, 1993; Butler et al., 2004; Fig 1c). The only obvious signs of the disease are lethargic behavior ultimately accompanied by decline and death of affected individuals. It is unclear whether clams with 100% CCH can reproduce, and it ap-

Figure 6. Phagocytosis in normal clam hemocytes (NCH) and cancerous clam hemocytes (CCH). Phagocytosis assay for freshly isolated NCH and CCH showed significantly reduced phagocytosis in CCH both in vivo and in vitro (Kruskall-Wallis: \( H = 106.6, P < 0.0001 \); all pairwise comparisons were significantly different from one another \( P = 0.05 \) using a Wilcoxon–Mann-Whitney test and an experimental-wide error of \( P = 0.0175 \).
pears that clams are most affected by the disease between 1 and 2 years of age, thus reducing the numbers of older clams at contaminated sites (unpubl. data). Thus, the degree of destruction of local clam beds attributable to cancer would not be obvious to local fishermen or regulators and has only been documented in detail using hemocyte, histological, and flow cytometric assays for clam beds in Massachusetts (Laevitt et al., 1990) and Canada (Morrison et al., 1993; McGladery, 2001; Delaporte et al., 2008). Our studies on p53 and mortalin family members in the soft shell clam have been reinforced by a recent report from a Canadian laboratory supporting the hypothesis that these genes are involved in development of CCH (Siah et al., 2008). Understanding the causes of naturally occurring diseases and correlating them with related molecular exposure mechanisms is a vitally important and virtually unexplored aspect of developing sustainable aquaculture for commercially important marine species.

Although aquaculture of soft shell clams is restricted to out-planting of seed clams and management of clam flats, this species could also be developed as a sentinel organism for other bivalves that are more frequently cultured in land-based facilities or open-ocean lease sites. Because the shells of soft shell clams have a wide gape, hemolymph can be aspirated from the pericardial cavity and gill tissue can be biopsied without sacrificing the donor. Worldwide, there are at least 15 commercially important shellfish species (e.g., Crassostrea virginica, Mytilus spp., and Ostrea edulis) that are impacted by a phenotypically similar proliferative disease. In these species the molecular basis for disease is less well understood, and it is much more difficult to biopsy hemolymph in bivalves that do not have the wide gape of the soft shell clam. Recent studies of several species of the mussel genus Mytilus have also identified variants of the p53-like family of proteins, including homologs for human p53, p63/73, and p63/73 DeltaN, and have demonstrated altered expression of these variants in the hemocytes of normal mussels compared to those with hemic neoplasia (Muttray et al., 2005, 2007, 2008; St-Jean et al., 2005; Banni et al., 2008). The studies have not addressed the idea that mortalin may also be involved in hemic neoplasia in mussels. Examination of soft shell clams as sentinel species to assess the prevalence of cancer in areas under consideration for bivalve culture could be accomplished indirectly using the simple biopsy we have described above. If exposure and transmission pathways turn out to be similar between bivalve species, as seems likely for Mytilus spp., high incidence of cancer in soft shell clams would be contraindicative for culture of other bivalve species in or near affected sites.

(c) Monitoring environmental health

Linkages between specific environmental toxins and human cancer are often difficult to demonstrate for lack of appropriate model systems. Van Beneden (2005) makes the important point that “bivalve molluscs are long-lived and sessile, with a filter-feeding strategy that exposes them to both suspended and soluble compounds.” Many heat shock proteins are expressed in response to environmental stressors, have differing threshold induction temperatures, and are important in coordinating the cell cycle and suppressing programmed cellular death (= apoptosis) (Feder and Hoffmann, 1999; Beere, 2001; Beere and Green, 2001; Buckley et al., 2001; Hofmann et al., 2002). Although evidence is not currently available, overexpression of mortalin, a member of the heat shock protein family, might be traceable to particular temperature or contaminant stressors (Buckley et al., 2001). This logic implies a direct line from the environment to a well-studied molecular clam cancer mechanism. Experimental studies of the clam cancer model are necessary to determine if such a relationship exists.

There is increased pressure to develop models and protocols for evaluating potentially hazardous compounds found in or released to the aquatic environment. As Van Beneden (2005) points out, “bivalves have low monooxygenase activity and as a result, compounds slowly accumulate and tissue burdens can serve as integrative indices of bioavailable contaminants.” Cancer in soft shell clam hemocytes is not currently utilized as an environmental indicator, although other bivalves (e.g., Mytilus spp. and Crassostrea spp.) have been investigated at the molecular level, through microarrays (Venier et al., 2003, 2006; Dondero et al., 2006; Jenny et al., 2007) and through detection of DNAstrand breaks (Halldorson et al., 2004), to follow specific cellular responses in challenged bivalve communities (e.g., metallothionein after metal exposure and heat shock proteins after different environmental stressors). Many monitoring and experimental studies have used mussels to establish timelines of exposure to xenobiotics and other anthropogenic perturbation such as eutrophication. In bivalves, variations in the activity of cytochrome P-450 enzymes and the detection of DNA strand breaks by the Comet assay are now regularly employed to quantify exposure to xenobiotics (Mitchelmore et al., 1998).

(d) Future research directions

Several important areas of research remain to be addressed as we continue to develop this versatile model. These include: (1) providing a more thorough understanding of the etiology of this disease in terms of exposure pathways or molecular mechanisms; (2) establishing soft shell clam cell lines for viral isolation and characterization and for in vitro studies of pathways of cell cycle regulation, apoptosis, etc.; (3) identifying the tissue source for cancerous clam hemocytes; and (4) cloning the soft shell clam genome and/or developing high-throughput microarrays for the detection of soft shell clam genes that are differentially expressed during the transition between normal and cancerous
cells and after environmental insults (Gracey and Cossins, 2003; Denslow et al., 2007).

(1) Disease etiology. The causes for onset of clam leukemia are unclear, but evidence exists for both contaminant and viral mechanisms. Without a more comprehensive idea of the genes that change in expression during the transition from NCH to CCH, correlated with measurements of particular environmental or viral factors that might promote such transitions, precisely determining the etiology of clam hemocyte cancer or similar hemic neoplasias in other bivalves will continue to be difficult.

Because we can induce cancer in otherwise normal clams by inoculation with hemolymph (lysed CCH) from clams with this disease (Fig. 5), we are in a unique position to observe changing profiles of p53 interacting proteins as NCH develop this mortalain-based disease. Given the development of antibodies to authentic clam proteins that are upregulated or downregulated by clam p53-like family proteins, the yeast two-hybrid screen and western blotting could be used to follow the initiation and progression of clam cancer. We already have antibodies to clam mortalin, and in such an experiment we would expect mortalain to increase in expression as hemocytes develop the disease. As other proteins are identified that are regulators of p53 or transcriptional targets for it, more comprehensive studies of this kind should be possible. Human studies and the demonstration of conserved molluscan homologs for human upstream regulatory and downstream transcriptional targets for it, more comprehensive studies of this kind should be possible. Human studies and the demonstration of conserved molluscan homologs for human upstream regulatory and downstream transcriptional target genes in the archeogastropod *Lottia gigantea* (http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html) suggest that Mdm2 (Chang et al., 1993), ribonuclease reductase (Tanaka et al., 2000), and BCI family members (Chipuk et al., 2004) among several other proteins would vary in expression (unpubl. data). Zero-time expression of Mdm2, ribonuclease reductase, and BAX proteins could be determined and then compared with values obtained after inoculation with hemolymph (minus CCH) from diseased clams and initiation of cancer in host normal clams. During progression of cancer, we expect that Mdm2 and BAX protein levels would fall to zero, since p53 will be retained in the cytoplasm and will be unable to transcribe the genes for these proteins. Similarly, molluscan homologs for human heat shock and other stress proteins could be assessed in normal clams that are experimentally exposed to environmental stressors to determine—based on their differential expression—which of these is the most likely to initiate the disease.

(2) A Bivalve cell line. Our development of the first primary cell cultures and of non-immortalized cell lines from CCH is a promising beginning. The limited lifespan of human cells in culture has led to the use of treatments that allow cells to achieve more passages. The lifespan of human cell lines can be extended by transfection with viral genes that produce products that sequester tumor suppressor molecules like p53 and retinoblastoma (Rb). In these cultures some cells will occasionally acquire mutations that make them immortal and even tumorigenic (Masters, 2000). The intriguing feature of CCH is that although they do not appear to be immortal, they are already tumorigenic. Although CCH maintained in vitro in our study were healthy and mitotic, possible refinements of our culture medium may be necessary to increase growth rates and to develop an immortalized cell line. However, if a virus is involved in this disease, the lifespan of CCH may already be extended via suppression of p53 transcription. Viral modification of the functions of CCH might be directly or indirectly involved in suppressing the activities of wild type p53 in complexes with over-expressed mortalain that sequester p53 in the cytoplasm. If this is the case, CCH may already be an immortalized cell line as defined for human cells in suspension culture (Masters, 2000)!

(3) Determining the source tissue for clam cancer cells. Soft shell clams with hemolymph containing 100% CCH could be exposed to 5-bromo-2′-deoxyuridine (BrdU) and will incorporate this thymidine analog into replicating DNA. By using an anti-BrdU antibody it may be possible to identify tissues with rapidly dividing cells in tissue sections of larger clams or in cleared whole mounts of smaller clams (perhaps in the tissues suggested by Cuénot, 1891). Candidate tissues with high levels of mitotic cells might be further characterized using quantitative polymerase chain reaction or western blotting to search for molecules known to be upregulated (e.g., clam mortalain and other stress-related proteins and p53 family members) or downregulated (e.g., Mdm2, BAX) during transition to the disease state. Tissues with high mitotic rates and expected expression levels for relevant genes might be cultured to determine whether they will yield cells with features characteristic of CCH that have been identified in the present study.

(4) Bivalve genome sequencing. Gene-specific cloning has been accomplished for many cell cycle related and other genes in bivalves (e.g., Kelley et al., 2001; Muttray et al., 2005, 2007, 2008; St-Jean et al., 2005; Bonni et al., 2008). An international consortium is sequencing the oyster genome (*Crassostrea gigas*; Hedgecock et al., 2005), and genome sequencing projects are underway for other molluscs (*Crassostrea virginica* and the mussel *Mytilus californicus*; see GenomeSeek database [Genamics, updated 2008]. The only completed genome is for the archeogastropod *Lottia gigantea* (http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html). Undoubtedly, completion of a bivalve genome will be of great advantage to bivalve aquaculture and pollution monitoring as groups of genes are identified that may determine disease susceptibility, tolerance to environmental stress, and mechanisms to manipulate growth (Saavedra and Bachère, 2008).
2006). To develop a more thorough understanding of both the etiology and disease pathology of clam hemocyte cancer, a full accounting of the genes that reflect similar diseases in humans could be very useful in disease diagnosis, treatment, and prevention, which are among the most significant variables in aquaculture. In addition, with a full catalog of the genes available, clam hemocyte cancer might turn out to be even more important as a preclinical model where exposure pathways or molecular mechanisms are shared with human diseases (Rabinowitz et al., 2005).

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