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DEVELOPMENT OF TECHNIQUES FOR IN VITRO PRODUCTION OF BIOACTIVE NATURAL PRODUCTS FROM MARINE SPONGES

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SUMMARY

Marine sponges are the source of many bioactive compounds with therapeutic potential. A critical issue in the drug development strategy for marine natural products is ensuring an adequate supply of compounds for clinical use while protecting the source organism and its habitat from over-exploitation. One approach is the development of techniques for in vitro production of bioactive compounds. Replicative cultures of the sponge *Teichaxinella morchella* have been established. Techniques for monitoring sponge cell responses to growth factors by semi-automated microtiter plate assays were developed, including the sulforhodamine B method for total protein and a Hoechst 33342 DNA dye-binding assay. The identity of cell cultures stimulated to divide by vertebrate growth factors and lectins was verified by the in vitro production of stevensine (odiline), a compound which is characteristic of *T. morchella*. Cultures retained their ability to synthesize stevensine after doubling, which demonstrates the feasibility of in vitro production of bioactive, sponge-derived natural products.

Key words: sponge, *Teichaxinella morchella*, Axinellida, stevensine, odiline, bioactive, natural products, cell culture, growth factors, phytohemagglutinin, cryopreservation

INTRODUCTION

Terrestrial plants and microorganisms have been an important source of natural products used in the development of drugs. During the past two decades, research on marine invertebrates and algae has demonstrated that marine organisms are also a valuable source of new and diverse chemical compounds with clinical potential. Over the past ten years alone, greater than 5,000 chemical compounds have been reported from marine organisms, particularly from marine sponges (Ireland et al., 1993) . A critical issue in the drug development strategy for marine natural products is ensuring an adequate supply of compounds for clinical use while protecting the source organism and its habitat from over-exploitation (Cragg et al., 1993; Rubin and Fish, 1994). Thus, alternative methods for the large-scale production of marine sponge-derived bioactive compounds are needed. Alternatives include chemical synthesis, aquaculture, and in vitro production of compounds.

The objective of our research is to establish cell lines of bioactive marine invertebrates that can be used as models to study in vitro production of bioactive metabolites and the factors which control expression of production, and ultimately, to produce bulk amounts of the bioactive products in vitro.

MATERIALS AND METHODS

Sample collection. The shallow water sponge *Teichaxinella morchella* (Phylum Porifera, Class Demospongiae, Order Axinellida, Family Axinellidae), which produces the antitumor compound stevensine (Albitzi and Faulkner, 1985; de Nanteuil et al. 1985, Wright et al., 1991), is our primary test model. Samples were collected by scuba from south Florida and the Bahamas.

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Dissociation and selective cell enrichment. Samples were prepared immediately after collection, as described by Pomponi and Willoughby (1994). The sponges were cut into fragments of approximately 1 cm^3 in artificial sea water (ASW). The fragments were rinsed in sterile calcium- and magnesium-free artificial seawater (CMF) (Spiegel and Rubenstein, 1972), then further minced with scissors or scalpels. The pieces were placed in CMF (10:1 CMF:tissue vol) for 10-20 minutes, and then gently forced through sterile gauze to release cells. After the cell suspension was filtered through 70 µm mesh nylon to remove cell aggregates and debris, the filtrate was concentrated to 10^6 - 10^8 cells/ml by centrifugation at 300 *g* for 5 minutes. Archaeocytes were separated from other cell types by layering 2 to 4 ml of the crude cell suspension on a discrete gradient (15-30-45-60%) of osmotically adjusted Percoll/CMF and centrifuging at 400 *g* for 10 minutes. The bands at the Percoll interfaces were collected by aspiration with a pipet, rinsed by diluting with CMF, and concentrated by centrifuging (5 min at 300 *g*). Viability was assessed by trypan blue exclusion.

Sponge Cell Culture Medium (SCCM). Medium 199 (M199) (Gibco BRL 11044-013) or Iscove's MDM (Gibco BRL 21056-015) were selected as basal media. Osmolality was increased to 1000 mOsm by addition of NaCl. The pH was adjusted to 8.1 and stabilized by addition of 5 mM Trizma buffer. Fetal bovine serum was added at 5%, but was eliminated from experiments testing the effects of vertebrate growth factors. Addition of antibiotics was adjusted according to experimental requirements (Pomponi and Willoughby, 1994). Rifampicin (1.16 mM) is used in all culture media to control bacterial contamination. To control fungal contamination in long-term maintenance of cultures, ketoconazole or amphotericin B (Fungizone) is added to the medium.

Cryopreservation. Archaeocytes (10^7 - 10^8 cells/ml) were suspended in a solution of 20% fetal bovine serum and 2.3 mM rifampicin in Sponge Cell Culture Medium (SCCM). An equal volume of CMF containing 15% DMSO was added slowly to the cell suspension, and the mixture was dispensed into 1 or 2 ml plastic cryovials. All solutions were sterile and the entire procedure was done on ice. Vials were cooled at a slow, controlled rate (1° C/min) using Nalgene cryo containers at -70° C for 4 hours. Samples were then stored at -140° C or in liquid nitrogen vapor.

Establishment of primary cultures. Cryopreserved cells were thawed rapidly in a 50° C water bath, then rinsed in CMF. Cells in were inoculated into T-25 flasks (Falcon Primaria) or multiwell plates containing SCCM at densities of 10^5 - 10^6 cells/ml and incubated at 20° C in air.

Microtiter plate assays for monitoring cultures. The sulforhodamine B (SRB) assay for measurement of protein (Skehan et al., 1989) was modified to quantify cell number of sponge archaeocytes. Cells were fixed with 80% trichloroacetic acid (final concentration, 16%), for 30 minutes at ambient temperature, followed by an additional 90 minutes at 4° C. This procedure fixes the cells and causes them to adhere to the plates. A series of five water rinses was followed by a 1 hr incubation with SRB (0.8 g/100 ml 1% acetic acid). After five 1% acetic acid rinses, the plates were air dried. The stain was then solubilized with 10 mM tris base (100 µl per well) and dispersed by shaking the plate on a gyrorotary shaker for 5 minutes. Absorbance was read at 570 nm.

To quantify and monitor synthesis of DNA in cultures, a microtiter-plate assay using the nucleic acid probe, Hoechst 33342, was developed. Cultures were incubated in Hoechst 33342 (4 mM) for 1 hour. The stain was excited at 355 nm and fluorescence was read at 468 nm (Perkin-Elmer Luminescence Spectrometer LS-50).

Monitoring cultures for production of bioactive metabolites. An analytical HPLC technique to detect small (25 ng) quantities of stevensine was developed to analyze small volumes of cells and media from *T. morchella* cell cultures. A sponge cell extract was prepared by adding 300 µl of methanol to the cell pellet (~10^6 to 10^8 cells). The pellet was macerated with a spatula and the mixture allowed to steep overnight at -20° C. The cell pellet extract was filtered through a cotton plug and rinsed two times with 300 µl aliquots of methanol. Filtered extracts were concentrated under a stream of nitrogen and then reconstituted in 100

to 150 µl of HPLC eluent. The HPLC analytical method utilizes a reverse phase separation on a Vydac C-18 large pore Protein and Peptide Column (10 µ, 4.6 mm x 250 mm). The column is run in isocratic mode with water-acetonitrile-acetic acid (85:15: 0.75) as the mobile phase and a flow rate of 1 mL/min. Stevensine can be detected by UV absorbance at 230 nm and is well resolved from other extract components under the analysis conditions. Stevensine is quantified by comparison to a standard curve.

RESULTS

Development of a primary culture. An in vitro model of the bioactive sponge, *Teichaxinella morchella,* was developed to determine the feasibility of in vitro production of the bioactive metabolite, stevensine. Archaeocytes are stem cells that are necessary for attachment, aggregation, and differentiation into other cell types (De Sütter and Buscema, 1977). Since most of the other sponge cell types are terminally-differentiated, archaeocytes were chosen for these studies. We had reported previously that the antibiotic, 5-hydroxytryptophan, is localized in the archaeocytes of *Hymeniacidon heliophila* (Sennett et al., 1990). On this basis, we hypothesized that enriched cultures of archaeocytes would be capable of proliferation and differentiation into metabolite-producing cells.

A major problem was the lack of sufficiently sensitive techniques to monitor the effect of different experimental variables. The number of available cells was insufficient for some established procedures, and the standard method of hemocytometer counts of viable cells was labor-intensive and time-consuming. For these reasons, the use of semi-automated procedures was assessed. A modification of the SRB assay was adapted to monitor subtle changes in protein concentrations in sponge archaeocyte cultures. This provided a rapid and sensitive procedure to quantify protein and to correlate total protein with cell number. Based on the successful implementation of the SRB assay, the Hoechst 33342 microtiter plate assay was developed, using the fluorescent probe to quantify DNA and monitor DNA synthesis in cultures. The assay correlated well with sponge cell number in the range reported herein. These semi-automated assays proved valid for the analysis of multiple combinations of media components and growth factors and obviated the need for manual cell counts of hundreds of samples per assay.

The next challenge was to obtain cultures of dividing cells. We reported previously that phytohemagglutinin (PHA) (1.5% by volume) stimulated cell division in cultures of the sponge *Hymeniacidon heliophila* (Pomponi and Willoughby, 1994). *Teichaxinella morchella* cells stimulated with PHA divided within 36 hours, as measured by direct cell counts (Fig. 1) and DNA synthesis (Fig. 2).

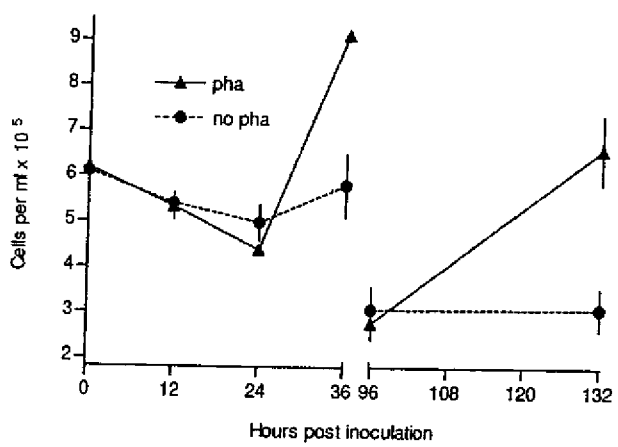

Fig. 1. PHA-induced growth of *Teichaxinella morchella* cells. Cultures were incubated in SCCM with and without 1.5% PHA. PHA-stimulated cultures (solid line) doubled after 36 hours, as measured by direct cell counts. Cell numbers in unstimulated controls (dashed line) did not increase. After 96 hours, subcultures were treated with PHA, and doubled after 36 hours. Control cultures showed no increase. (Mean +/- S.E., n= 4).

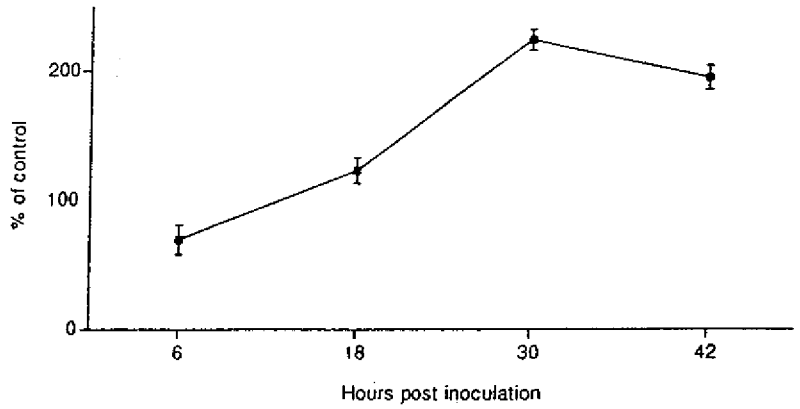

Fig. 2. PHA-induced DNA synthesis in *Teichaxinella morchella* cells. Cultures were treated as described in Fig. 1. DNA was measured by the Hoechst 33342 microtiter plate assay. At 18 hours post-inoculation, the DNA content of PHA-stimulated cultures increased. The relative DNA content of PHA-stimulated cultures reached a plateau at 30 hours, at greater than 200% of the DNA content of the untreated control. (Mean +/- S.E., n = 5).

Because of the success obtained in culturing vertebrate cells in serum-free media, a preliminary growth factor supplement (GFS) consisting of several growth factors, organic nutrients, a lectin (PHA), a transport factor (transferrin), and hormones was formulated and added to the sponge cell culture medium (using Iscove's MDM as the basal medium). The GFS as well as some of its constituents stimulated cell division in *Teichaxinella morchella* cultures (Fig. 3). As individual supplements, PHA and bovine pituitary extract (BPE) were most effective in stimulating growth in relation to the control.

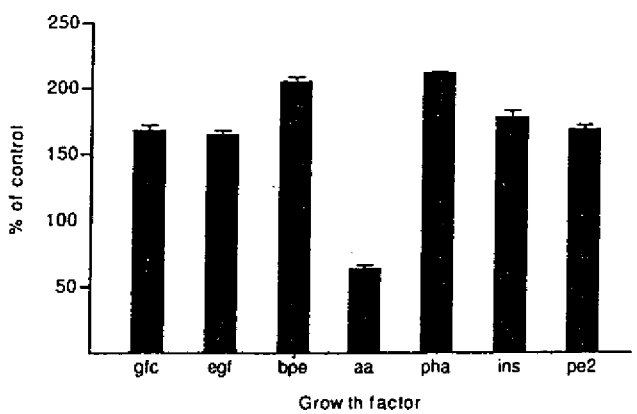

Fig. 3. Effect of growth factors on *Teichaxinella morchella* cultures after 48 hours incubation in Iscove's MDM. Data are plotted as percent control of protein concentration, as measured by the SRB assay. Control cultures (horizontal dashed line) received no growth factors. GFS, growth factor supplement (EGF, epidermal growth factor, 10 ng/ml; BPE, bovine pituitary extract, 25 µg/ml; AA, arachidonic acid, 10 µM; linoleic acid, 0.2 µM; cholesterol, 5 µM; phosphoethanolamine, 10 µg/ml; PHA, 1.5%; transferrin, 5 µg/ml; INS, insulin, 10 µg/ml; PE2, prostaglandin E2, 50 ng/ml; hydrocortisone, 50 nM, retinol acetate, 0.3 µM). Concentrations of components of GFS tested individually are the same as in the complete GFS. (Mean +/- S.E., n = 5).

In vitro production of the bioactive metabolite. To determine the minimum number of cells required for the analysis, serial dilutions of archaeocytes recovered from cryopreservation were analyzed. Cell counts ranged from a high of 1×10^8 cells to a low of 2.5×10^6 cells. Stevensine could be detected in all of the samples analyzed. Therefore 2.5×10^6 cells was chosen as the minimum number of cells required for the analysis.

To determine if cultures which underwent cell division in response to PHA continued to produce the bioactive metabolite, PHA-stimulated and control (unstimulated) cultures were subsampled for analysis of stevensine concentration after 36 hours and 8 days in culture. The PHA-stimulated cultures doubled within the first 36 hours, but no doubling occurred in the control cultures (without PHA) (Fig.1). A total of 6×10^7 cells from each culture were analyzed. After 36 hours, both PHA-stimulated and control cultures contained the same amount of stevensine per cell, indicating that cells which divided continued to produce the bioactive metabolite (Fig. 4). After eight days in culture, the PHA-stimulated cells contained more stevensine per cell than at the beginning of the experiment, while the non-stimulated cells contained less (Fig. 4). These results indicate that the cells retain their ability to synthesize stevensine after doubling, and demonstrate that sponge cells will respond to mitogenic stimuli without disruption of biosynthetic pathways.

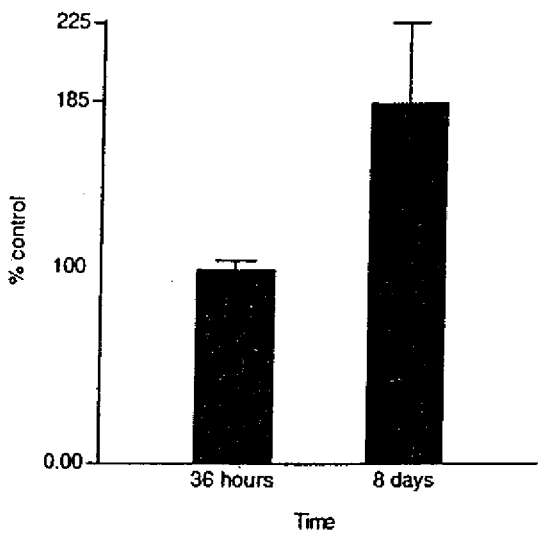

Fig. 4 Concentration of stevensine per cell in cultures of *T. morchella* incubated in PHA, indicated as percent of control. One population doubling occurred within 36 hours in PHA-stimulated cultures; control cultures did not divide. After 8 days, PHA-treated cultures showed an increase in stevensine concentration per cell.

DISCUSSION

Methods have been developed for the replicative culture of sponge archaeocytes stimulated to divide by vertebrate growth factors and the lectin phytohemagglutinin. Development of a continuous sponge cell line, however, has not yet been achieved. Our continuing research focuses on optimization of culture conditions for sustained growth and metabolite production of either normal or transformed cells. Stimulation of cultures with precursors to the bioactive compound may enhance production of the metabolite. Unfortunately, the biosynthetic pathways for most marine-derived natural products are not well understood, so the selection of precursors can be difficult. A sponge cell line, once established, may provide an important opportunity for studying biosynthesis of sponge metabolites.

Authentication of cell lines is as important in marine invertebrate cell culture as it is in vertebrate cell culture. Some previous reports of cell lines of sponges and other marine invertebrates have been subsequently determined to be protozoan or fungal contaminants (personal communication, Dr. Baruch Rinkevich, National Institute of Oceanography, Haifa, Israel). The identity of cultures of *T. morchella* is verifed by their ability to produce stevensine, which constitutes approximately 0.54% of the sponge wet weight and is strongly suspected to be from the sponge and not from a microbial symbiont. DNA fingerprinting is now used in most cell repositories to authenticate cell lines. Methods for the consistent extraction, amplification and sequencing of sponge genomic DNA have been established (Kelly-Borges and Pomponi, 1994) and may be used to authenticate cell lines through amplification and sequencing of partial DNA sequences from cultured cells and source sponges.

Results of research to date indicate that in vitro production of bioactive sponge metabolites is feasible, and that sponge cell culture models can be used to study factors which control expression of production of the bioactive metabolites. Further research using sponge cell culture models may lead to enhanced synthesis of bioactive metabolites in culture, as well as the development of genetically engineered models using sponge cells or other expression vectors.

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