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IN VITRO PRODUCTION OF MARINE-DERIVED ANTITUMOR COMPOUNDS

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The sustainable supply of marine-derived bioactive compounds to meet demands for pre-clinical and clinical evaluation is a major consideration in drug development strategies. It is also a concern to environmental resource managers, particularly since conservation of genetic resources may preclude harvesting as a bulk supply strategy. Our research addresses this need by focusing on in vitro production of antitumor compounds through cell culture of the source organisms. Our objectives are: to establish cultures of bioactive marine invertebrates that can be used as models to study in vitro production of antitumor compounds and the factors which control expression of their production; to provide bulk supplies of these compounds through in vitro production; and to produce new structural analogs via manipulation of culture conditions.

We have established primary cell cultures of two bioactive marine invertebrates: the sponge Teichaxinella morchella which produces the antitumor compound, stevensine (Albitzi and Faulkner, 1985), and the tunicate Ecteinascidia turbinata from which the antitumor ecteinascidins have been isolated (Rinehart et al., 1990; Wright et al., 1990). Cultures were initiated using techniques developed in our laboratory for the dissociation and selective enrichment of cells known to contain the bioactive metabolites: archaeocytes in T. morchella (Pomponi and Willoughby, 1994; Pomponi et al., 1997), and stem cells in the embryos and larvae of E. turbinata. Cells are inoculated into microwell plates (10^5 to 10^6 cells/ml) in the appropriate nutrient medium. Sponge cell culture media are based on commercially available media (Medium 199 or Iscove's MDM). Osmolality is increased to 1000 mOsm by addition of NaCl. The pH is adjusted to 8.1 and stabilized by addition of 5 mM Trizma buffer. Tunicate medium is based on a formula by Kawamura and Fujiwara (1995). Rifampicin (1.16 mM), streptomycin, or penicillin are used to control bacterial contamination, and ketoconazole or amphotericin B (Fungizone) is used to inhibit fungal growth in long-term maintenance of cultures.

We have developed multiwell plate assays to measure effects of growth-regulating compounds on DNA and protein synthesis. To quantify and monitor synthesis of DNA in

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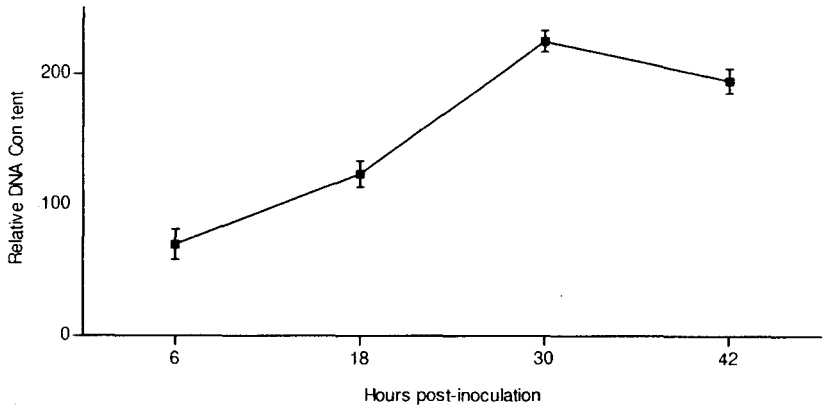

Figure 1. PHA-induced DNA synthesis in Teichaxinella morchella cells, expressed as percent of control. Cultures were incubated in Medium 199 with 1.5% PHA. DNA was measured by the Hoechst 33342 microwell plate assay. At 18 hours post-inoculation, the DNA content of PHA-stimulated cultures increased. 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). (From Pomponi et al., 1997).

cultures, we have developed a multiwell assay using the nucleic acid probe, Hoechst 33342 (Pomponi et al., 1997). Protein synthesis is measured using a modification of the sulphorhodamine B (SRB) multiwell plate assay (Skehan et al., 1989).

Since a cell line has not yet been developed, our research is focused on optimization of culture conditions for sustained growth, cell division, and bioactive metabolite production by normal cells. A medium supplement consisting of several growth factors, organic nutrients, a lectin (phytohemagglutinin [PHA]), a transport factor (transferrin), and hormones was formulated (Pomponi et al., 1997). Phytohemagglutinin stimulated DNA synthesis (Fig. 1) and cell division (Fig. 2) in T. morchella cultures. The supplement, as well as some of its constituents (epidermal growth factor, bovine pituitary extract, PHA, insulin, and prostaglandin E2) stimulated protein synthesis (Fig. 3A) in T. morchella cultures. Epidermal growth factor and insulin stimulated DNA synthesis in E. turbinata cultures, however, in the presence of fetal bovine serum (3%), DNA synthesis is inhibited.

To determine if Teichaxinella morchella cultures which underwent cell division in response to phytohemagglutinin (PHA) continued to produce the bioactive metabolite stevensine, PHA-stimulated and control (unstimulated) cultures were subsampled for analysis of stevensine concentration after 36 hours and 8 days in culture. Microanalytical

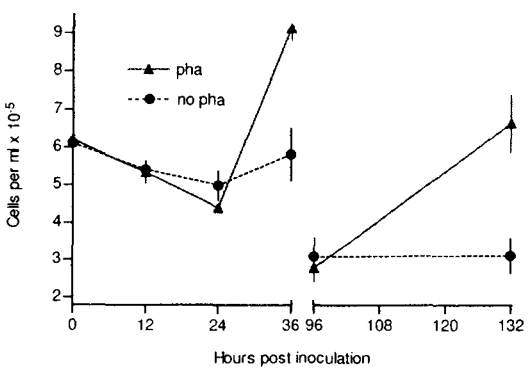

Figure 2. PHA-induced growth of Teichaxinella morchella cells. Cultures were treated as described in Fig. 1. PHA-stimulated cultures (solid line) doubled after 36 hours, as measured by direct cell counts. Cell numbers in unstimulated controls (dashed line) did not increase. After 96 hours, subcultures were stimulated with PHA, and doubled after 36 hours. Control cultures showed no increase. (Mean ± S.E., n= 4). (From Pomponi et al., 1997).

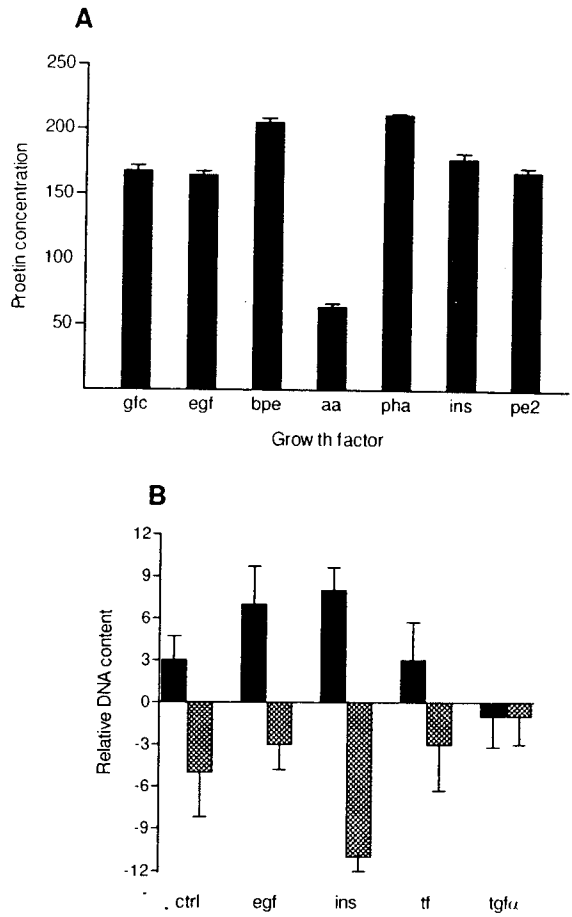

Figure 3. (A) Effect of growth factors on protein synthesis in Teichaxinella morchella cultures after 48 hours incubation in Iscove's MDM. Data are plotted as percent of control. Control cultures (horizontal dashed line) received no growth factors. gfc, growth factor cocktail (consists of egf, epidermal 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, phytohemagglutinin, 1.5%; tf, 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 gfc tested individually are the same as in the complete gfc. (Mean ± S.E., n = 5). (B) Effect of growth factors on DNA synthesis in Ecteinascidia turbinata cultures after 48 hours incubation in Kawamura medium, with (black bars) and without (patterned bars) 3% fetal bovine serum. Cell-free controls were subtracted from data shown. Concentrations are as listed for Fig. 3A. tgf-a, transforming growth factor-a, 10 ng/ml. (From Pomponi et al., 1997).

HPLC techniques were developed to monitor in vitro production of stevensine (Pomponi et al., 1997). 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).

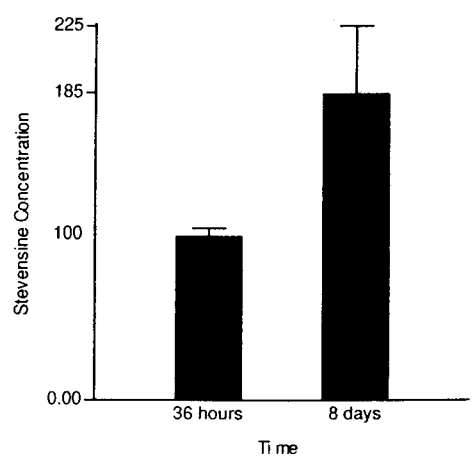

Figure 4. Concentration of stevensine per cell in cultures of T. morchella incubated in PHA, expressed as percent of control. One population doubling occurred within 36 hours in PHA-stimulated cultures; control cultures did not divide. After 8 days, PHA-treated cultures showed an increase in stevensine concentration per cell. (From Pomponi et al., 1997).

After eight days in culture, the PHA-stimulated cells contained more stevensine per cell than at the beginning of the experiment (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.

In summary, methods have been developed for the primary culture of sponge and tunicate cells stimulated with vertebrate growth-regulating compounds and the lectin phytohemagglutinin. Development of a continuous sponge or tunicate cell line has not yet been achieved. In vitro production of the sponge-derived antitumor compound stevensine demonstrates, however, that this approach is feasible for further development as a biological model for studying the factors which control expression of production of marine-derived antitumor compounds.

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