CELL CULTURE FROM SEA CUCUMBER (*CUCUMARIA FRONDOSA*) DERMIS

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The mutable mechanical properties of echinoderm catch connective tissues are modulated by resident cells that modify interactions between collagen fibrils. We recently identified in the dermis of *Cucumaria frondosa* two cell-derived factors that regulate tissue compliance in live specimens *in vitro* (Koob *et al.*, *J. Exp. Biol.* 202; 2291-2301, 1999). One factor from the inner dermis caused compliant specimens to become stiff in bending tests; the other factor from the outer dermis plasticized stiff specimens. A variety of cell types populate the dermis, including fibroblast-like cells, cells containing distinct assemblages of cytoplasmic granules, nerve fibers and neurosecretory cells. Since extracts from which these factors were purified originated from whole dermis, the cells that produce and release each factor could not be determined, although the stiffener was found predominantly in the matrix-rich inner dermis while the plasticizer was found primarily in the outer dermis. Therefore, we set out to establish conditions for culturing isolated dermal cells in order to explore which cell type produces each factor and what mechanisms regulate their secretion. Since culture of cells from echinoderm connective tissues has not been reported as far as we are aware, our objective was to establish conditions for reproducibly obtaining and culturing cells from the dermis of *Cucumaria frondosa*.

Initial attempts to isolate cells by standard enzymatic treatments failed, probably because the tissue is densely collagenous and incubations had to be carried out at low ambient sea water temperatures. However, two morphologically distinct cell types emerged from untreated full-thickness dermal explants that were cultured in L-15 medium supplemented to 0.5 M NaCl, 50 mM $MgCl_2$, 10 mM $CaCl_2$ and 10 mM KCl, and further buffered with 20 mM MOPS (Fig. 1). Cells appeared either suspended in the medium or attached to the plate within 24 hours *in vitro*. Round cells with podia migrated out of the tissue and remained in suspension. Elliptical cells containing numerous, large cytoplasmic granules migrated out of the tissue and attached to the culture plate.

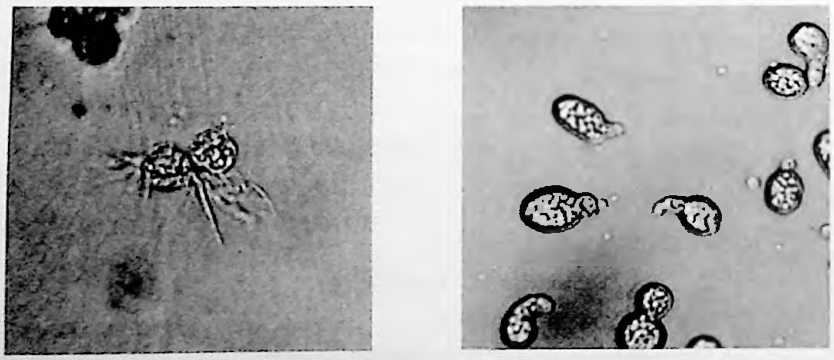

Figure 1. Cells migrating out of cultured sea cucumber dermis. Left panel: Cells with podia in suspension. Right panel: Cells with granules attached to the substratum.

To examine the conditions that facilitated reproducible culture of sea cucumber dermis cells, a method for measuring the number of viable cells was essential. The MTT cell viability assay was chosen since it has been effectively used on cultures of mollusk mantle cells (Poncet *et al.*, *Mar. Biotechnol.* 2; 387-398, 2000). The assay is based on measuring active cellular dehydrogenases in living cells. Before applying this assay to attachment and proliferation experiments, preliminary tests established that the MTT assay could be reliably employed to measure the number of viable sea cucumber cells in culture as well as the optimal conditions for the assay. Cultured cells metabolized MTT at 13°C producing the appropriate color product. Boiled cells did not metabolize MTT. The extent of color development was directly proportional to the number of cells and incubation time.

In order to establish optimal conditions for obtaining large numbers of representative cells, the effect of media composition on the rate of cell migration out of explants was examined. Four media were compared: L15 supplemented with sea water salts (modified L-15); MOPS buffered artificial sea water supplemented with MEM amino acids and vitamins (ASW); MOPS buffered EGTA/ASW lacking calcium supplemented with MEM amino acids and vitamins (EGTA/ASW); sea water supplemented with MEM amino acids and vitamins (SW). Identical sized full-thickness dermis specimens were cultured in 24-well plates for 2, 4, 6 or 8 days at 13°C. At each time point, cells were collected from the medium and plate, combined, centrifuged and assayed for cell number with the MTT assay. The results from two animals are shown in Fig. 2. Modified L-15 was the optimal medium for cell emergence, while ASW promoted cell migration almost as well. Sea water supplemented with MEM was less permissive to cell emergence. Few cells emerged from explants in calcium-free EGTA/ASW with MEM. While the rate at which cells migrated out of the tissue varied between the two animals, the differences between media were similar.

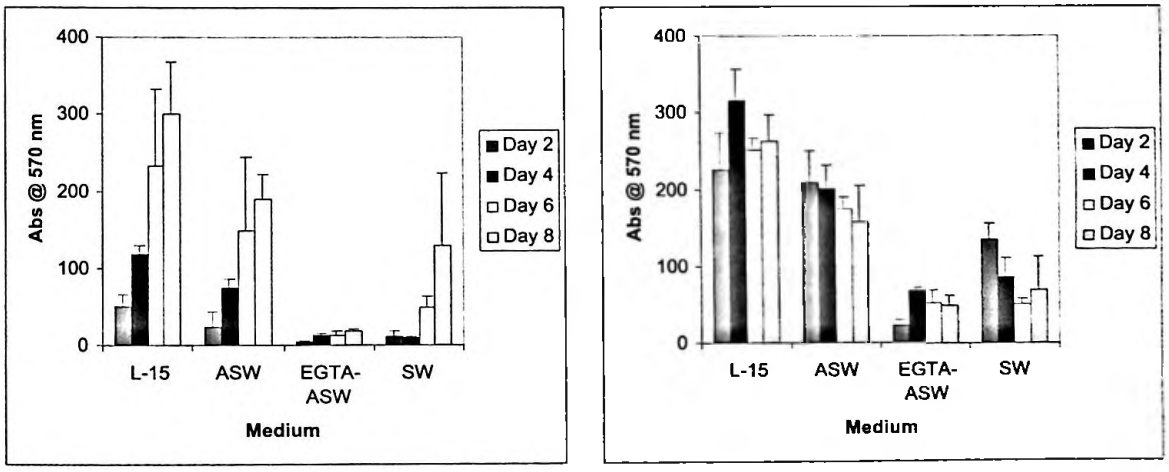

Figure 2. Effects of medium composition on the rate of emergence of cells from cultured dermis explants as measured by the MTT cell viability assay. Measurements on specimens from two animals. The results are presented as the absorbance of the color product in the assay. Values are means of 3 wells +/- S.D.

The potential role of substratum on the attachment of isolated dermis cells was assessed by plating cells on 24-well plates that had been pre-coated with extracellular matrix macromolecules. The following coatings were tested: isolated dermis collagen fibrils; dermis collagen fibrils stripped of non-covalently bound macromolecules; purified dermis microfibrils; and bovine gelatin. In addition, conditioned medium obtained from cell/explant cultures by centrifugation was used to coat wells. Cells that had migrated out from full-thickness dermis explants cultured for 4 days were collected by aspiration of the medium. A portion of this initial cell suspension was reserved, and the remainder was passed through a cell strainer. A portion of the strained cells was reserved, and the remaining cells were collected by centrifugation at 600 x g x 10 min. Each of these cell preparations was plated on coated culture dishes and cultured for 24 hr. The number of cells attached to the plate was measured with the MTT assay following removal of the suspended cells by aspiration. The results are presented in Figure 3 below and are expressed as the color product from the MTT assay (left panel) and the percentage of cells attached as compared with the total number plated (right panel). Cells attached to all wells including the untreated plastic. However, cell attachment varied according to coating. While cells effectively attached to all of the dermis extracellular matrix macromolecules, the microfibrils were most effective at facilitating cell attachment.

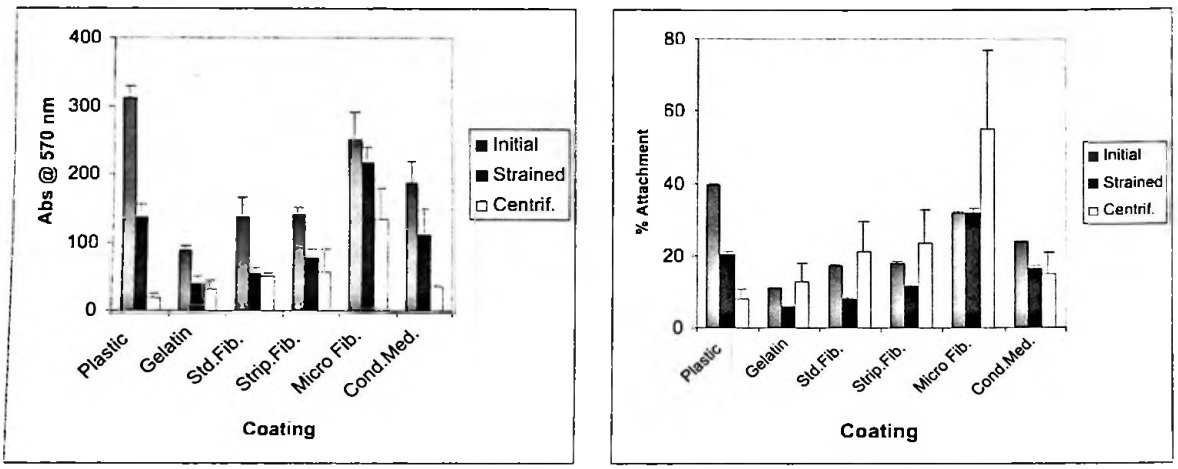

Figure 3. Effect of substrate on sea cucumber dermis cell attachment *in vitro*. The left panel shows the number of cells attached as represented by the color product in the MTT assay. The right panel shows the percentage of plated cells that attached. Plastic – untreated tissue culture plastic; gelatin – bovine gelatin; Std.Fib. – isolated, native collagen fibrils from the dermis; Strip. Fib. – dermis collagen fibrils extracted with 3 M guanidine; Micro Fib – purified microfibrils from the dermis; Cond. Med. – conditioned medium from dermis cells. Values presented are means of 4 wells +/- S.D.

To determine whether cells that emerged from dermis explants proliferated *in vitro*, and to examine the effects of media composition on proliferation, cells were collected from cultured explants and plated on 24-well dishes. The medium with suspended cells was removed after 24

hr, the attached cells were cultured in the various media described above. After 1, 3, 5 and 7 days, the number of cells attached to the plate plus the number of cells in the medium were measured. The total number of cells as measured by the MTT assay at each time point is shown in Figure 4. The number of cells appeared to decline during the first 5 days in culture in all media except EGTA/ASW. From day 5 to day 7, the number of cells increased in the L-15 and SW media. The number of cells in the EGTA/ASW remained constant throughout the 7 days in culture.

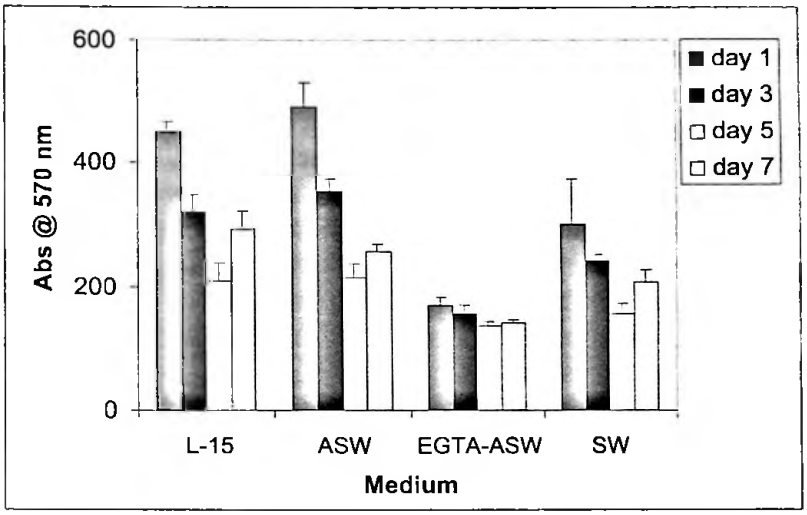

Figure 4. Effect of medium composition on sea cucumber dermis cell proliferation *in vitro*. Both attached cells and cells in suspension were measured. Values shown are means of 4 wells +/- S.D.

The identity of the cells that emerge from sea cucumber dermis *in vitro* remains an open question. These cells appear to harbor high concentrations of cytoplasmic granules, similar to the putative regulatory cells in the dermis. Further analyses using antibodies to the regulatory factors and immunocytochemistry will facilitate identification of these cells. In addition, characterization of expressed proteins will determine whether one or more cell types are responsible for biosynthesis of the extracellular matrix, particularly collagen and proteoglycans, and other macromolecules that mediate collagen fibril interactions, e.g., stiparin and stiparin inhibitor. The ability to culture cells from the sea cucumber dermis will effectively allow future investigation of the mechanisms involved in regulating secretion of factors that mediate reversible changes in the shape and mechanical properties of this echinoderm catch connective tissue.

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