Long-term Culture of Epithelial Cells Derived from Axolotl (*Ambystoma mexicanum*) Embryos

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Introduction

The Mexican axolotl (*Ambystoma mexicanum*) has considerable research potential in developmental biology, cell physiology, and cell differentiation because of its known mutants (5). A first step into the investigation of the cellular and molecular basis of cell lethality in autonomous cell-lethal mutant axolotl embryos (5) requires the long-term cultivation of axolotl embryo cells past the essential metabolite stores of embryonic cells.

The long-term cultivation of amphibian cells has been worked out for many anurans (3, 7). Except for one urodele cell line (TVI) (7) the number of reports on the long term cultivation of urodele cells is sparse and there is no report on the long term cultivation of axolotl cells in the literature. The culture requirements and methodologies for the long-term culture of urodeles in general and axolotls specifically still need to be established.

This report describes the first long-term cultivation of epithelial cells derived from normal axolotl prehatching embryos. The procedure depends on the establishment of primary explant embryo cultures that select for vigorous epithelial cell growth and the subsequent subcultivation of epithelial cultures not as dissociated single cells but as pieces of epithelial sheets (50 to 200 cells/piece).

Methods and Materials

Primary Explant Culture: Nine individual stage 36-38 (1) normal axolotl prehatching embryos from the Indiana University Axolotl Colony (Bloomington, Indiana) were explant cultured in individual culture dishes. Individual embryos preincubated in 20% Steinberg's solution (S) with antibiotics (Penicilln-Streptomycin 5 mcg/ml each and Fungizone 2.5 mcg/ml at pH 7.4) from 2 to 24 hours in their jelly coats were sequentially immersed (20 seconds) in 1% hypochlorite (Chlorox) in 20% S; 20% S with antibiotics; 70% Ethanol in 20% S and then washed 3 times in 20% S with antibiotics. Mechanically dejellied embryos were anesthetized in 1/50000 MS-222 (ethyl-m-aminobenzoate, methane sulfonic acid; Aldrich Chemical Co., Milwaukee, WI in 100% S with antibiotics (Gentamicin 50 mcg/ml), skinned (tail, torso, and head), and the yolky abdomen removed. The dissected embryos were transferred to complete culture medium: 55 parts Leibovitz's L-15, 25 parts doubly distilled H_2), 20 parts Fetal Bovine Serum (Flow Laboratories, Lot –29101338, McLean, Virginia), 0.02 U/ml insulin (Sigma), 1 ug/ml thyroxin (Sigma), 50 mcg/ml Kanamycin, 2.5 mcg/ml Fungizone at pH 7.8 $\pm$ 1. The embryos were minced into 0.5 to 1 mm diameter pieces and the pieces transferred to wet 35 mm tissue culture dishes (Corning) or glass petri dishes, prewashed with complete medium, with a minimal amount of culture medium. After a 2 hour incubation in a humid atmosphere at 25°C, 0.5 ml of culture medium was added without disturbing the attached explants. At 24 hours the culture medium volume was increased to 1.5 ml. Every third day the cultures were fed with 1.5 culture medium.

Subculturing: The primary or subsequent subcultures were washed with 2 ml 75% L-15, washed again with 2 ml Ca^{+} and Mg^{2+} free PBS and then incubated from 30 to 60 minutes with 1 ml dissociation medium containing 1.8 U/ml Protease type IX

from *Bacillus polymyxa* (Sigma) in Ca^{2+} and Mg^{2+} free PBS, at 25 °C in a rotatory shaker (60 rev/min). The dissociation medium was collected, its volume doubled with complete medium and centrifuged (setting 3) on a desk-top centrifuge (IEC) for 2 minutes. An equal volume of complete medium was added, and the pellet was disrupted into small pieces (approximately 50 to 200 cells per piece) with repeated pipeting with a small bore pipette. The pieces were centrifuged as described above and the pellet resuspended in (1.5, 3, or 6 ml) complete medium. The pieces were plated in 35 mm tissue culture dishes (Corning) or 35 mm glass petri dishes at 1.5 ml per dish. The cultures were incubated in a humid atmosphere at 25 °C and fed 1.5 ml complete medium every three days.

Morphology and Cell Packing Density: Cell cultures were observed directly (phase), indirectly (video recordings, Panasonic) or by standard histological techniques. The mean cell packing density was determined by enumerating the number of cells occupying 0.1 mm^2, on photographs of at least 4 separate areas.

Proliferation: The large cell size and monolayer growth of these cells made it possible to determine the mitotic index (MI) and duration of mitosis (T_M) quite accurately. The MI was calculated as the ratio of mitotic cells (M) to total cells (Interphase (I) and mitotic cells) expressed a % (M/I + M) x 100). Cell cycle time (T) was estimated from MI and T_M according to the relationship $T = \log_e 2\ T_m/MI$ (8).

Results and Discussion

Primary Explant Culture: Eighty five percent to 95 percent of the embryo explants showed cell outgrowths by 24 hours in culture. Proliferation began by day 5, and beating heart cells and cilia cells, extensive nerve fiber outgrowths, and muscle cell differentiation were observed during the 56 to 78 days the primary cultures were maintained. Although there was a gradual decrease in the number of explants with cell outgrowths (day 36, 55%; day 50, 27%) by day 25 each culture had at least one proliferating monolayer epithelial outgrowth demonstrating homogenous cell morphology and an increase in cell packing density and epithelial sheet area. The origin of the epithelial cells is unknown. Cell morphology, culture behavior, and the fact that the embryos were skinned indicates that the epithelial cells are not epidermal cells.

Subculturing: Preliminary attempts to subculture primary cultures having epithelial sheets with the following dissociation media were unsuccessful: 0.25% trypsin (GIBCO); 1.8 units/ml protease (Type IX, Sigma); 0.125 trypsin and 0.9% protease (all in phosphate buffered saline diluted to 200 m Osm (PBS) without Ca^{2+} and Mg^{2+}, pH 7.2 at 25 °C. Typically, in 1:1 and 1:2 split ratios, from 1 to 9% of the cells attached. Similar dissociation conditions on 18 day primary cultures resulted in 20 to 50% cell attachment. There was a gradual degeneration of cells and by 18 days in culture few cells remained attached to the substrate. Mitosis was only observed in patches of confluent cells that platted as aggregates. These observations were used to successfully subculture epithelial sheet cultures by dissociating the epithelial sheets into pieces of approximately 50-200 cells each by the procedure outlined in the materials and methods.

Subcultured individual cells and epithelial sheet pieces attached with 24 hours. All the single cells eventualy degenerated and most epithelial sheet explants lost their confluency and eventually degenerated. About 10% of the epithelial sheet explants retained confluency and showed vigorous growth. These epithelial sheets increased both in cell packing density and area (Table 1) and were morphologically indistinguishable from the primary epithelial sheets. Cultures were subcultured when the majority of epithelial sheets achieved 30-50 $x10^4$ cells/cm^2. These epithelial sheets were cultured for 305 days and subcultured 4 times (78, 173, 230, and 290 days after culture initia-

TABLE 1
Cell Packing Density and Areas of Epithelial Sheets

Days in culture	Days after subculturing	Low packing density	High packing density	Mean packing density	Area of sheet
60	primary culture	15.5 ±2.5 a)	30.3 ±4.3	22.9 ±8.5	29 mm^2
173	95	—	—	55.7 [±12.0]	39 mm^2
186	13 b)	—	—	6.4 [±1.3]	.9 mm^2
203	30 c)	8.8 ±1.0	19.2 ±4.8	15.0 ±6.5	11 mm^2

The data is the mean ± SD a) of at least 4 separate counts of the number of cells occupying 0.1 mm^2. The packing density is expressed as 10^4 cells/cm^2. b) and c) represent data on the same epithelial sheet.

tion), before they were lost due to an inadvertant switch to a different lot of fetal bovine serum. Cultures were negative for both mycoplasmal contamination (direct agar technique) (4), and bacterial contamination (microscopy) (2). This same lot of fetal bovine serum did not support the initiation of new epithelial sheets from primary cultures.

Morphology and Cell Packing Density: During the long term cultivation of these epithelial cells there was no change in cell morphology or any obvious change in chromosome number (Figure 1). The highest cell packing density observed was 55.7 x 10^4 cells/cm^2. This was probably their saturation density because proliferation was occurring simultaneously with cell detachment with no net change in cell packing density.

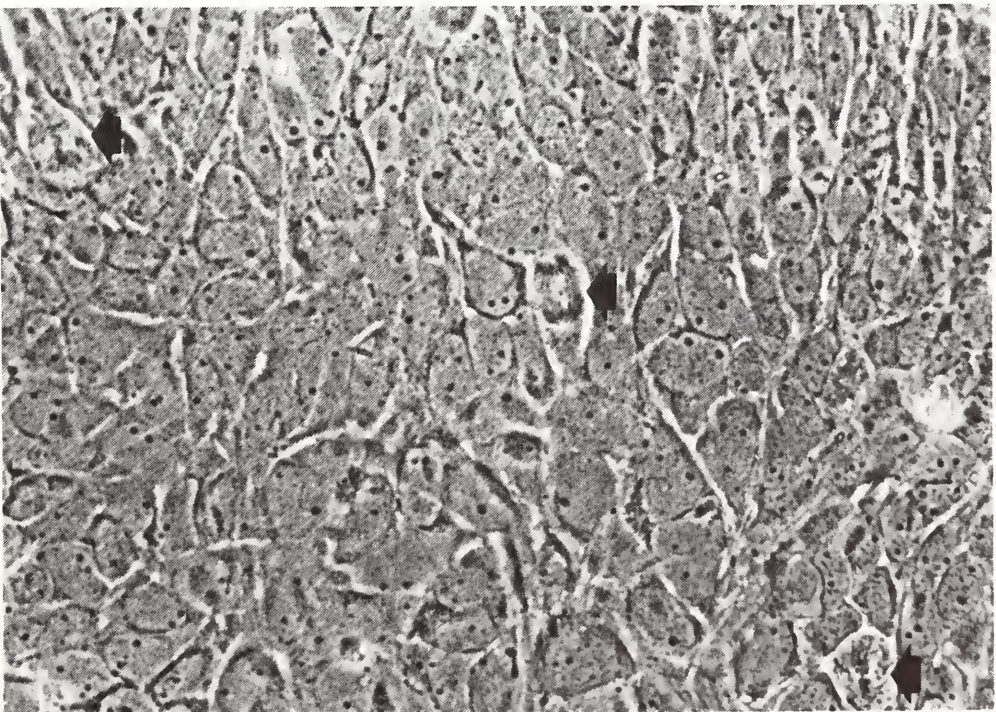

FIGURE 1 *Phase micrograph of an epithelial sheet 211 days in culture showing typical cell morphology, low and high cell packing densities and several mitotic cells (arrows). (193 x magnification)*

Proliferation: Proliferation was evident at 5 days in primary cultures and 2 to 5 days after subcultivation. Proliferation was evident at all cell packing densities throughout the epithelial sheets. Mitosis was only observed among confluent cells. The MI, T_M and T for epithelial cells during the first and second subculture (Table 2) remained

TABLE 2
Mitotic Index, Duration of Mitosis and Estimated Cell Cycle Time in Epithelial Cell Cultures

Days in culture	Mitotic Index (MI)	Duration of Mitosis (T_M) (minutes)	Estimated Cell cycle time (T) (hours)
80-109 (first subculture)	1.2 ±.8	80 ±25	46
179-215 (second subculture)	1.1 ±.3	80 ±18	50

The cell cycle time was estimated using the relationship $T = \log_e 2\ T_M/MI$ (8). Data represents the mean ± SD of at least 9 measurements during the culture periods indicated.

constant. Spot checks of the MI and T_M during the fourth subculture (data not shown) gave similar values. These values lie within the range usually reported for amphibians (7). The observation that during the long-term cultivation of these epithelial sheets the morphology of epithelial cells and sheets remained constant, that the culture behavior of the epithelial cells and sheets remained constant, and that there was no dramatic change in chromosome numbers indicates the possibility that these cells probably did not transform. These observations are consistent with the observation that chromosome number of amphibian cell lines tend to be stable and amphibian cell lines are frequently epitheliod and have no intrinsic senescenee phase (6, 7). The axolotl epithelial cell sensitivity to fetal bovine serum and requirement for cell-cell contact for active proliferation may explain why the establishment of long-term cultures of axolotl cells has been difficult. In this light the first long-term cultiviation of epithelial cells from pre-hatching axolotl embryos, described in this study, is important for the eventual establishment of cell lines from normal and mutant axolotls.

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