Development of Isolation and Cultivation Method for Outer Root Sheath Cells from Human Hair Follicle and Construction of Bioartificial Skin

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Abstract

It is difficult to obtain sufficient healthy skin for coverage of a wide area of skin wound. In the skin, an additional population of living epithelial cells is located in the outer root sheath (ORS) of hair follicles.[1),2)] ORS cells should be a good source of epithelium because they are easily obtainable and patients do not have to suffer from scar formation at donor sites.

We modified ordinary primary culture technique for the purpose of solving such problem that epithelial cells have a low propagation and easy aging during culture periods. First of all, we improved primary cultivation methods. In the ordinary primary culture, average yield of human ORS cells was 2×10^3 cells/follicle by direct incubation with trypsin (0.1%)/EDTA (0.02%) solution for 15 min at 37℃ but we could obtain about 6.5×10^3 cells/follicle by two step enzyme digestion method with dispase (1.2 U/ml) and trypsin (0.1%)/EDTA (0.02%) solution. So we could achieve three times higher primary cultured ORS cell yield. Secondly, we could obtain total 2×10^7 cells in serum free medium and even more total 6×10^7 cells in modified E-medium with mitomycin C-treated feeder cells during 17 days.

Using the cultured ORS cells, and we could make bioartificial skin equivalent *in vitro* and concluded that ORS cells were progenitor cells for skin epithelial cell.

Materials and methods

Isolation of ORS cells from hair follicles was carried out in three methods. In the firstgroup, hair follicles became loose by incubation in 1.2 units/ml dispase (Gibco BRL, Grand Islands, N.Y., U.S.A.) in PBS for 16 h at 4℃ followed by incubation with trypsin (0.05%)/EDTA (0.02%) solution for 15 min at 37℃. In the second group, direct incubation with trypsin (0.1%)/EDTA (0.02%) solution for 15 min at 37℃ was performed. And in the third group, explantation cultivation method was performed after incubation in trypsin (0.05%)/ EDTA (0.02%) for 15 min on the type collagen coated tissue culture dish in serum free medium. Primary cultures were performed in modified E-medium or in Keratinocyte-Serum Free Medium (K-SFM, Gibco BRL) added calcium ion up to 0.15 mM. Modified E-media used ORS cells culture that co-cultured with mitomycin C-treated NIH J2 3T3 feeder cells[3].

The *in vitro* construction of a bioartificial skin began by casting the cell/collagen mixtures as dermal equivalent onto the 3 mm porous polycarbonate membrane of the culture insert (MILLICELL, Millipore, USA). Fibroblasts were gently stirred with a solution containing seven volumes of 5 mg/mL collagen solution (CELL GEN, KOKEN, Japan), two volumes of 5×DMEM, and one volume of 0.05 N NaOH containing 2.2% sodium bicarbonate and 200 mM HEPES buffer solution[4]. After the culture of dermal equivalent for 2 days, culture medium was aspirated to leave the gel surface exposed, ORS cells were applied on the surface. The construct was submerged for two days and then to develop the multilayered artificial skin the whole system was lifted at the air-liquid interface. After 10 days, the bioartificial skin was fixed in 10% neutral buffered formalin for 2 h at 4℃. Fixed samples were embedded in paraffin and the 5 mm-sectioned paraffin ribbons were stained with hematoxylin and eosin.

Results and discussion

We modified ordinary primary culture method. We found out that the two step enzyme digestion was very useful method which can give a highly viable primary cell yield(Fig 1).

Effect of different medium on the growth of human ORS cells. They were the modified E-medium used with mitomycin C-treated NIH J2 3T3 feeder cells and K-SFM added calcium ion. Considering growth curve and cell morphology, serum supplementation and co-culture with feeder cells in modified E-medium system had a promoting effect on cell

proliferation and maintain normal morphology. Soluble factors and cytokines secreted by NIH J2 3T3 feeder cells inhibited differentiation and stimulated proliferation of ORS cells(Fig 2).

Fig. 3A clearly shows polygonal ORS cells growing out from the ORS of a plated hair follicle in 7 days after explantsculture in K-SFM. Fig. 3B showed ORS cells colony formation and grew in a cobblestone pattern and process conspicuous nuclei in 6 days after enzyme digestion primary cultivation in K-SFM. Fig. 3C shows ORS cells(O) co-cultured with mitomycin C-treated NIH J2 3T3 feeder cells(J). After 5-6 days in culture, single ORS cells or groups thereof surrounded by post-mitotic feeder cells become visible.

The morphology of the bioartificial skin is shown in Fig. 4. The construct was fixed for light microscopy after 10 days culture at the air-liquid interface. Histological examination shows epidermal layer (E) with stratification (S) and dermal substrate (D) containing collagen with cells. The stratified epidermal layer was composed of approximately 5 ~ 6 ORS cells layers. The epidermis consists of stratified cell layers formed by continuous proliferation and differentiation of cells from basal ORS cells to stratum corneum layer.

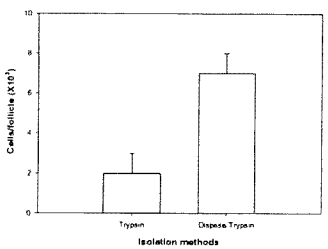


Fig. 1. The isolation yield of ORS cells by different enzyme digestion method.

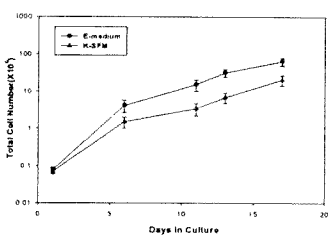


Fig. 2. Effect of different medium on the growth of human ORS cells.

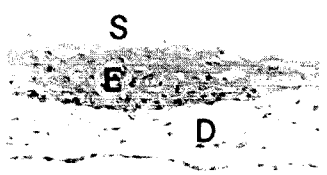
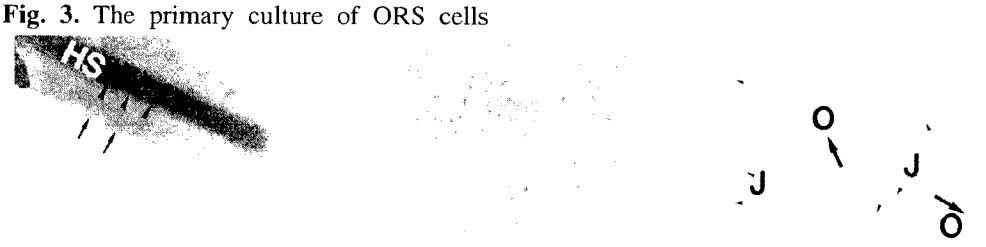


Fig. 4. Phase - contrast photograph of a bioartificial skin cultured *in vitro*

Fig. 3. The primary culture of ORS cells



(A) Explantation method (B) Enzyme digestion method (C) Co-culture method

Conclusion

In our work, the two step enzyme digestion was very useful method because many viable ORS cells can be recovered without damage. The primary cell yield of human ORS cells was average 2×10^3 cells/follicle by conventional direct incubation with trypsin and we could obtained about 6.5×10^3 cells/follicle by two step enzyme digestion with dispase and trypsin. And we found that the primary culture method and type of medium influenced not only cell growth rate but also cell morphology. We confirmedthat structure of bioartificial skin made by ORS cells or made by keratinocytes of adults skin was very similar to real skin structure. So it was concluded that ORS cells are progenitor cells of the skin epithelial cells and they represent a source of easily and repeatedly available keratinocyte.

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