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ORIGINAL PAPER

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Enzyme digestion to isolate and culture human scalp dermal papilla cells: a more efficient method

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Abstract In this study, we show a more efficient method for isolation and cultivation of dermal papilla cells from hair follicles of human scalp skin. The dermal partments of low hair follicles were pulled out from cutaneous fat and the bulb epithelium was teased out from the fibrous sheath with attached dermal papilla by applying gentle pressure with the tip of an occal forceps. When these fibrous sheathes were entirely digested into isolated cells by collagenase D but the dermal papillae were justly to be digested, collagenase D was discarded and the dermal papillae were isolated completely out from the resuspension solution by repeated low-speed centrifugation and transferred to another dish for free-floating culture. This procedure markedly simples the steps of isolated dermal papilla operation and relieves the laborious tension. Furthermore, dermal papillae could be isolated on a large-scale and remained intact. After collagenase digestion, the dermal papillae showed very high adherent rate and quicker growth than that of microdissection, which suggests that the definition factor of dermal papilla cell migration was relaxed and some structure had been activated or exposed. The cells exhibited a multi-layer forming property and spread-out growth style. They showed positive with alcian blue, with toluidine blue O for different gradient pH and PAS, which was similar to the staining results of in situ dermal papilla. It suggests that the culture papilla cells still synthesize and excrete neutral and acid mucopolysaccharides. Our results demonstrate that the papilla cells in culture condition still remain the ability to synthesize the specific extracellular matrix components of in situ dermal papilla, which supports the concept that the dermal papilla cell, a highly specialized fibroblast, especially is involved in hair growth regulation.

Keywords Hair follicle · Dermal papilla cell · Cell culture · Histology

Introduction

The dermal component of the hair follicle is believed to play an important role in the development, reformation and maintenance of hair follicle and control of the hair cycle growth. In earlier reports, on the most-studied mammalian species, rats, Oliver [1–4] showed that dermal papilla (DP) was necessary for the induction and maintenance of epithelial differentiation in the vibrissa and possessed the ability to induce the formation of new follicular structures when associated in contact with glabrous epidermis and dermal sheath had a very close relation with DP because it could form a new DP to continue the whisker growth when DP or up to one third of the lower follicle was removed.

Since Jahoda and Oliver [5] successfully culture cells from isolated vibrissa DP in 1981, the papilla cells from hair follicles of human scalp skin [6, 7], beard and axillary hair [8, 9] or from murine vibrissa [10], sheep vibrissae [11] and rat pelage [12] are also passaged. Many characteristics of the cultured DP cells have been discovered [13–15]. These include a characteristic spread-out growth, flatten morphology, a low proliferative activity, easy senescence and the ability of forming multi-layered aggregation at cell confluence, which distinguishes from fibroblasts. Furthermore, it is most important that cultured rat DP cells, in particular, retain

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the ability to induce hair growth after microsurgical implantation into the ends of follicles that had their dermal sheath and papilla excised [15]. But the most commonly used method to obtain hair follicular papillae is the microdissection on excised tissue. However, microdissection is a laborious and time-consuming task, and depends on the skill of the operator. Some drawbacks, such as low products, uneasy adhesion, low-alive rate and poor growth-out from the papilla plants, have also been a formidable barrier to the study of the role of papilla cells in hair growth regulation. Recently, Warren [16] reported that the attacked rate remarkably increased after the freshly isolated papillae were treated with collagenase type IV and cell yields were improved considerably. Another article exhibited that, after removing the fascia and part of subcutaneous tissue to expose hair bulb as possible, the treatment of the low hair follicles with dispase was easy to isolate DP by microdissection, greatly facilitated the attachment of DP explants and improved its growth [17]. Reynolds [18] also demonstrated that dispase was helpful to isolate DP. But these methods still use microdissection and don't overcome the laborious and time-consuming drawbacks.

The size of the follicle and hair diameter is considered to correlate with the volume of DP. Papilla volume is in turn related to the ECM and the number of papilla cells. But the number of DP cells does not change during the hair cycle or during the lifetime of mammalians [19]. However, the ECM of DP changes in the feature and quantity during the hair growth cycle. In anagen, DP is composed of elongated stromal cells containing many organelles, suggesting active protein synthesis. These cells are embedded in an abundant ECM consisting of homogeneous flocculent material, rich in fibronectin, type IV collagen, laminin and proteoglycans, and also, in human DP, interstitial collagens [20–22], some enzymes are logically needed to digest fibronectin and/or type IV collagen. In telogen, the papilla cells have less cytoplasm and organelles than the anagen cells, which are a cytologically typical example of a more quiescent state. The papilla is decreased in size, primarily because of a reduction or loss in ECM, especially the glycosaminoglycan content such as chondroitin sulfate proteoglycan (CSPG) [10]. So these specific ECM components of DP are tightly coupled to the hair growth cycle. CSPG not only is highly hydrated molecules related to papilla volume, but also provides immune privilege to anagen hair follicles [23, 24]. Furthermore, the aggregative behavior of papilla cells is related to these proteoglycan [16].

There are different results among published reports on the nature of ECM components expressed by the mammalian papilla cells in culture [20, 25, 26]. Maybe the ability of DP cells to synthesize basement membrane collagen type IV was lost or replaced by the expression of types I and III. These differences may be partly due to the different medium, in which isolated DP were cultured [16]. Furthermore, DP cell mediums contained collagen type III, CSPG and heparan sulfate proteoglycan(HSPG). Immunohistochemical staining showed that the papilla cells in vitro continued to synthesize high levels of laminin, collagen type IV and CSPG [16].

In this study, we report a very high efficient method for isolation and cultivation of human scalp DP cells. After the bulb epithelium was gently squeezed out from the dermal sheath following incubation with 0.5%dispase for 16∼18 h, the dermal part of low hair follicles was digested by collagenase D for isolated DP. This method not only greatly simplified the operated steps of isolated DP, but also relieved the laborious tension. Furthermore, this procedure greatly facilitated the attachment of DP explants, improved its growth and increased cell yields. Finally, the papilla cells in vitro were positive stained with alcian blue(AB), toluidine blue O and AB-PAS, which resembles what has been described for DP in situ. It implies that the culture papilla cells still synthesize and excrete some specific ECM components as in situ DP cells.

Materials and methods

Isolation of DP by collagenase D

Normal scalp tissue was obtained from three dying young healthy men (aged 18–30 years) who were suffering from trauma, which obtained the agreement with their family member. Biopsies were uniformly taken from the occipital scalp region of men who didn't suffer from male-pattern alopecia or from any other diseases affecting the growth of hair and scalp. The species of biopsies were kept in D-Hanks at 4°C not over 48 h. The scalp skin was repeatedly rinsed by D-Hanks for three times, each for 10 min, then cut into strips about 0.3–0.5 cm in width, then cut off at the interface of dermo-subcutaneous fat with chippers. The subcutaneous tissue was incubated with 0.5% dispase (Sigma Chemical Co. St. Louis, MO) at 4°C for 16∼18 h. The dermal partments of low hair follicles were pulled out from cutaneous fat. Its epithelia were extruded out from the dermal sheathes by applying gentle pressure with the tip of an occal forceps or a microforceps. Then the dermal sheathes incubated in 0.2% collagenase D (Boehringer Mannheim, Germany) in DMEM medium (Sigma Chemical Co. St. Louis, MO) (containing 10% natal calf serum) at 37°C for 6∼8 h until the stalk of DP was digested under microscope control, when the fibrous sheath had been digested entirely and the papilla just began to be digested, the enzyme digestion should be stopped. After D-Hanks was added, the suspension was centrifuged for 5 min at 2,000 rpm, which was repeated for three times. The pellet was resuspended and centrifuged at low-speed about 200 rpm for 5 min and repeated for three times leaving dermal sheath fibroblasts in the supernatant for passage culture. The pellet was panned in culture dish with 10% calf serum DMEM and the supernatant were centrifuged at low-200 rpm for

5 min, which was repeated for three times. So DPs were completely isolated out from residue with low-speed centrifugation. The final DP pellet was resuspended without any isolated cells, transferred into a 25 ml-flask containing medium for explant culture in DMEM medium with 10% calf serum. The cultures were incubated for 5 days, then the medium was changed; thereafter, the medium was changed twice per week.

Human dermal fibroblasts (HDF) were initiated from explants of foreskin and scalp skin dermis using standard fibroblast culture techniques. Dermal sheath cells were also cultured from explants.

Microdissection of DP

Some anagen papillae were isolated with microdissection under a binocular stereomicroscope (magnification × 25) using a microforceps and a needle attached to 5 ml syringe, whose tip curved into about 90° was very useful for operation, according to Messenger's method. After the dermal sheath with attached DP was separated from the bulb epithelium and opened, the DP was extruded out by applying gentle pressure with the tip of the curved needle, and transected across its stalk, then transferred to a culture dish. Microscopic examination showed no residual epithelium left on the isolated papillae. Papillae were cultured in a 24-well plate for 5 papilla in each well or in a 25 ml flask for 25 papilla in each flask, in which some were free-floating, others were attached to a dish wall coated with or without rat-tail collagen. In some wells of free-floating culture, 10% natal calf serum DMEM was added; to some wells, little medium was added to define the DP's movement, and to others only natal calf serum was added. The medium was changed when cells were observed growing out of the explants, and then changed twice per week. Experiments were repeated at least in triplicate for five different subjects.

Histochemical staining

DP cells, dermal sheath cells, and dermal fibroblasts of scalp skin and foreskin were subcultured as usual. Passage 5 cells of DP, passage 10 cells of dermal sheath and dermal fibroblasts were plated on circle coverships in a 24-well plate, used for 1% Alcian blue (AB)(pH2.5), AB-PAS, H.E. May-Griunwald-Gemsa, and 1% AB (different gradient pH from 0.5 to 4.0) and 0.1% toluidine blue O (pH from 0.5 to 6.0). The cells at nearly confluence were fixed with Bouin's solution for 1 h, rinsed with 0.01 M PBS for three times, then treated with 0.2%Triton-100 for 20 min at room temperature and rinsed again. The following steps of these histochemical stain procedures were performed as usual except circle coverships were dried by an electric bulb and mounted in neutral balsam.

Immunohistochemical staining

At nearly confluence cells, the coverships were washed with 0.01 M PBS, treated with 0.2% Triton-100 for 30 min at room temperature, washed with distilled water for three times, then fixed with 100% ethanol for 5 min at room temperature and washed with 0.01 M PBS again. After being labeled with mouse anti-human vimentin, desmin (kindly provided by Dr. R. C. S. Kamaekers) and factor VIII related antigen antibody (Dako. Co.) at 4°C overnight, the coverships were incubated with biotinolated horse anti-mouse-IgG antibody and avidin-biotin complex (ABC Kit from Vector Co.) at 37°C for 30 min and 45 min, respectively, and finally diaminobezidin (DAB) was reacted. In the intervals of all incubation steps, washes with 0.01 M PBS were interposed.

Results

Characteristics of DP cells

Compared with other reports, we found that the DP isolated by microdissection was very hard to adhere walls when freely floating in 10% natal calf serum DMEM medium, even in little medium or in natal calf serum, or covered by a circle covership. Eight of 60 DPs were attacked. However, when a nylon membrane was covered on the free-floating DP, which still unattached at 5 days, but the cells could migrate out from DP to the nylon membrane. By another method, we firstly attached the DP on walls coated with rat-tail collagen, the cells did not grow out from the explants yet. The operation of microdissection needs good skill and is time-consuming, and the basement membrane of dermal papilla is still intact. However, a matrix digestion steps with collagenase D not only improved cell yields considerably, but also simplified the steps of operation and relieved labor tension. Treatment of low fibrous sheath with attached DP directly by collagenase D remarkably improved the frequency of explant outgrowth (generally over 99%). The most important point was to isolate the DP on a large scale because this method could obtain thousands of isolated DP at one time.

The DP after digestion was intact in spherical or ellipsoidal shape. It attached quickly within one day and the cells migrated immediately just overnight after plated (Fig. 1). On the first day, its attached rate was 96.5% (277/287), on the third day was 99.7% (286/287). However, in the following days, the rest unattached DPs could not adhere. The initial outgrowth of cells from the DP explants usually occurred within one day. These cells displayed flatten, polygonal morphology with several processes of various lengths extending from the edges of a striated cytoplasm containing pigments at the beginning. The striation and pigments were lost after seven passages in general. The cells

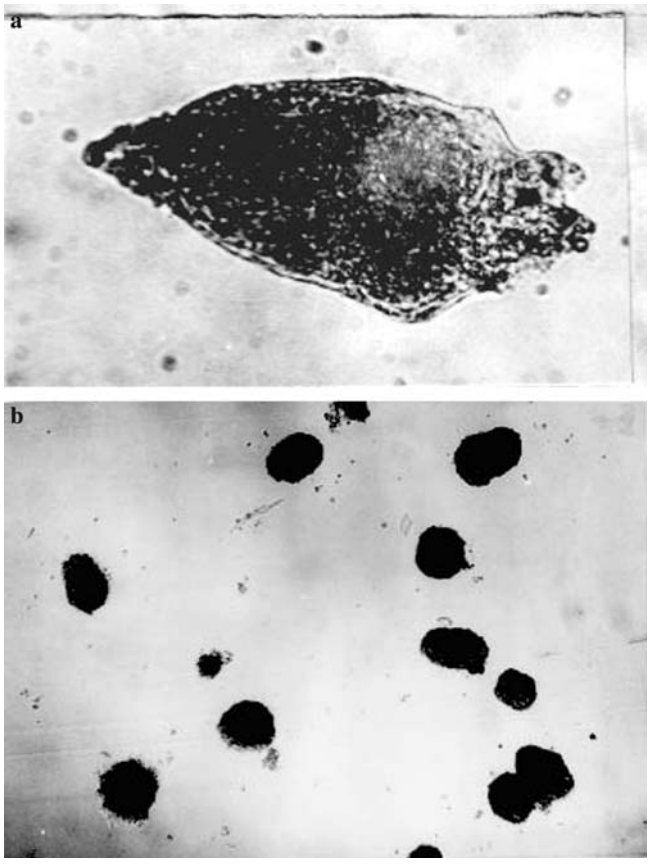

Fig. 1 Isolated dermal papillae: **a** control isolated by microdissection method. ×100; **b** digested out by collagenase D, cells had migrated out after plated for overnight. ×40

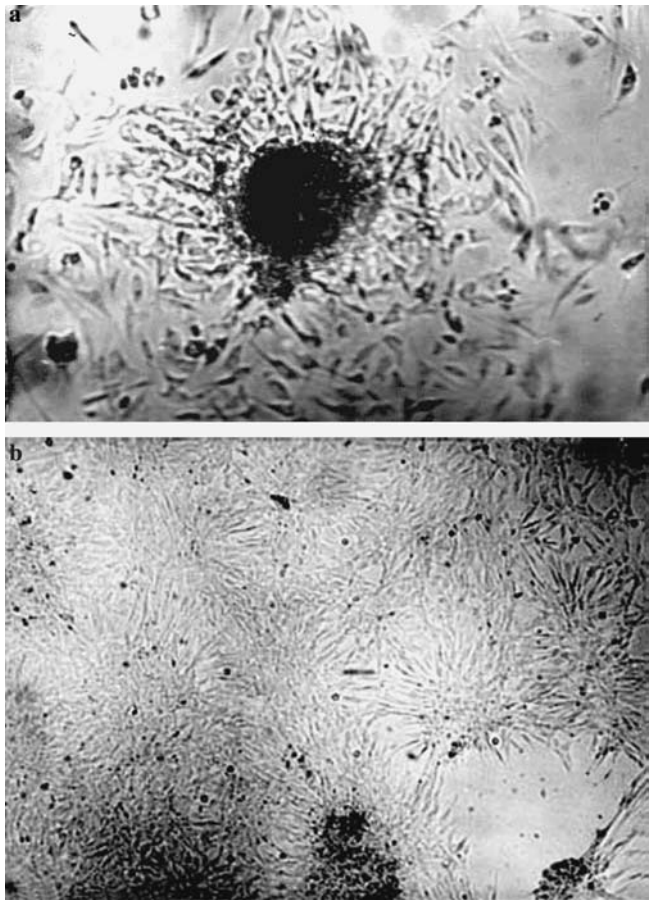

Fig. 2 The dermal papilla cells grew out like a sunflower. **a** ×100, **b** ×40

spread out from DP explants like sunflowers (Fig. 2). They showed in the appearance of spindle shape when they formed multilayered parallel arrays. These characteristics distinguished from dermal fibroblasts and dermal sheath cells. The papilla cells proliferated quickly and could be subcultured into passage 1 within 2 weeks after primary culture. The DP cells were generally spindle-shaped at low density and had a tendency to aggregate and clump into a multi-layered structure before confluence occurred. This aggregative behavior was more pronounced in first two subcultures and existed through over passage 7. In primary cultures, most of DPs showed a more typical fibroblastoid growth pattern, forming small branching parallel arrays of spindle-shaped cells, but they were larger and contained more cytoplasm than dermal fibroblasts, and a few of DPs displayed markedly aggregative behavior, and failed to grow to confluence throughout their life span. Three DP cell lines we established could be passaged over 15.

Dermal sheath cells mainly showed bipolar spindle-shape morphology, but did not display the aggregative behavior at confluence. At low cell densities, dermal sheath cells often exhibited a larger cytoplasm. With increasing cell numbers, dermal sheath cells formed into branching parallel array (Fig. 3). When confluence was reached, dermal sheath cells became very tight and their cytoplasm was contracted remarkably.

Histochemical staining

The cytoplasm of most DP cells showed blue when stained with AB (pH2.5). At the low pH (0.5), the cells were still stained with pale blue color. With increasing pH gradient, the blue became darker. When AB combined with periodic acid Schiff reaction (AB-PAS), some DP cell cytoplasm showed blue while other showed red, or in the same cell, one part of cytoplasm was blue while other was red. In multi-layer aggregates and clumps rich in abundant ECM, AB and PAS became stronger positive (Fig. 4). These results suggested that the DP cells contained and excreted acid and neutral mucopolysaccharides. When stained with low pH toluidine blue O, DP cell cytoplasm showed deep blue. With increasing pH gradient, DP cells became purple (Fig. 5).

Except showed positive when stained with AB, dermal sheath cells were negative for PAS and toluidine blue O. Dermal fibroblasts were all negative with AB,

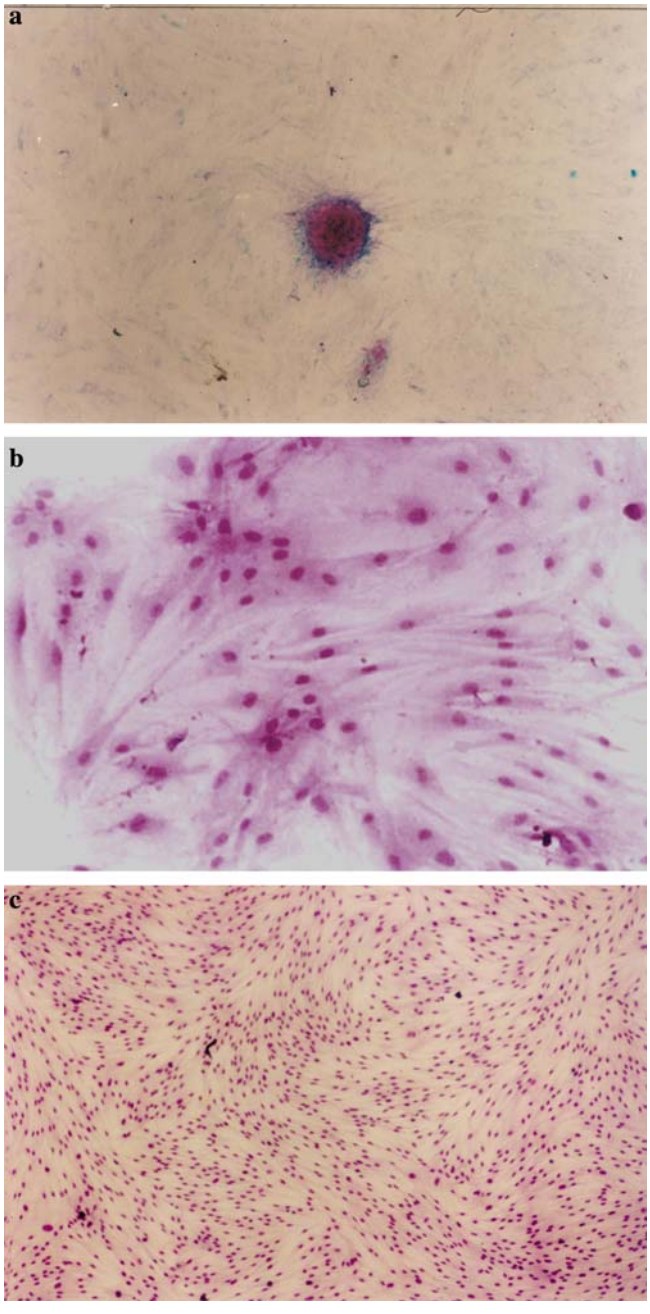

Fig. 3 **a** DP cells showed multi-layer clumps (AB-PAS) ×40; **b** Dermal sheath cells (May-Griunwald and Gemsa) ×100; **c** fibroblasts (May-Griunwald and Gemsa) ×40

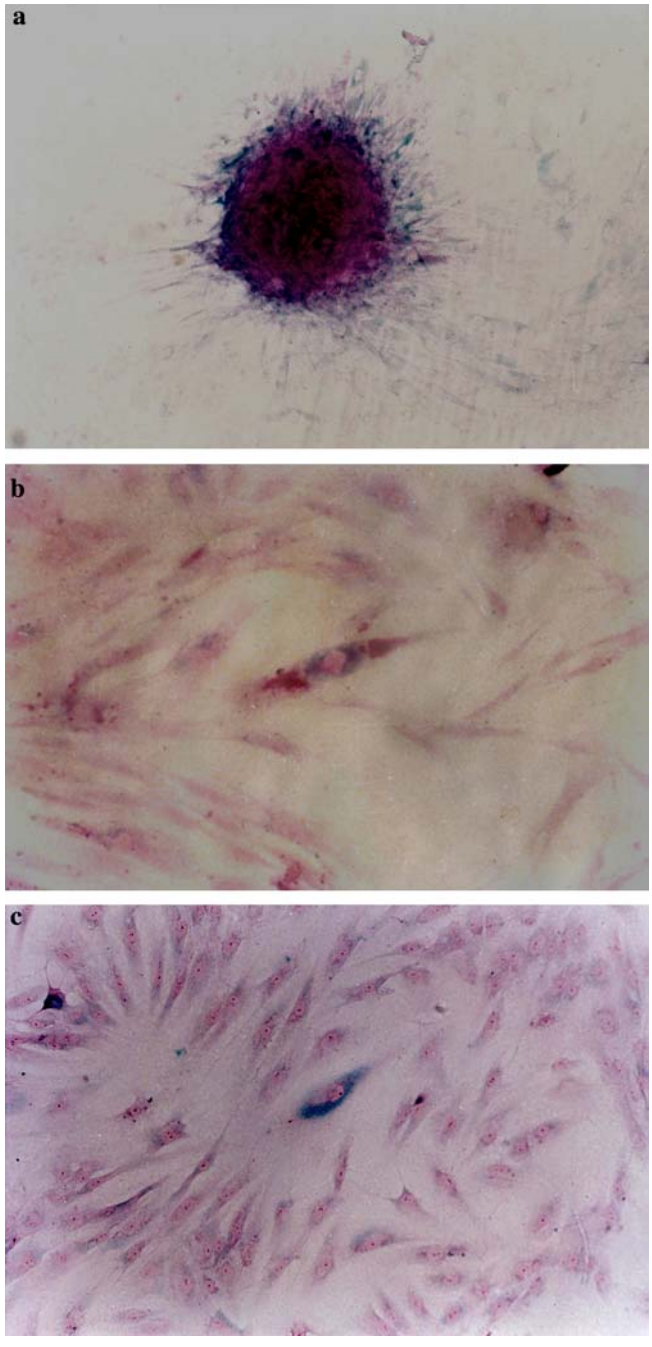

Fig. 4 **a** The multi-layer clumps of dermal papilla cells showed AB-PAS positive within and out of cytoplasm; **b** the same DP cells showed AB-PAS positive; **c** The dermal sheath cells showed alcian blue positive (AB-HE). ×100

PAS and toluidine blue O. The results showed as in Table 1.

Immunohistochemical staining

DP cells, dermal sheath cells and dermal fibroblasts from scalp skin and foreskin were all positive for vimentin. Except that a few of DP cells (passage 5) were weakly positive for factor VIII-associated protein (Fig. 6), most of DP cells, dermal sheath cells and fibroblasts were negative for factor VIII-associated protein and all negative for desmin.

Discussion

The results of the present study show that the method directly digesting the dermal partments of low hair follicles by collagenase D after dispase separates the junction between epidermis and dermis is markedly to

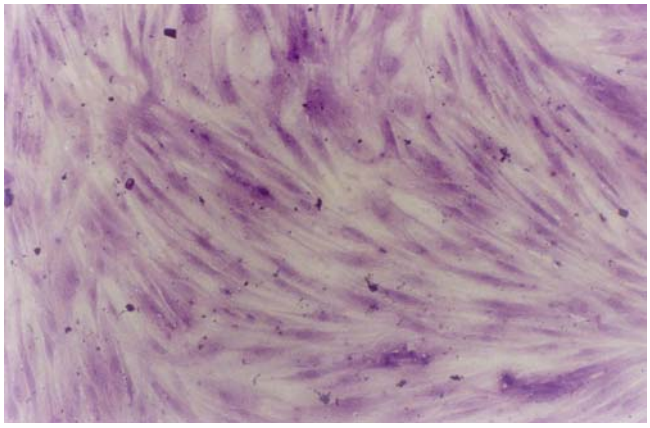

Fig. 5 The dermal papilla cells stained deep blue with tobuidine blue O (pH2.0) and became purple when pH went to 6.0. ×100

Fig. 6 Dermal papilla cell was positive with vimentin ×100

improve the isolation and growth of DP, and that the histochemical characteristics of DP cells are different from dermal sheath cells and fibroblasts.

Our method is to use dispase to separate the junction between epithelium and dermal sheath of low hair follicles in subcutaneous tissue and to use collagenase D to digest directly the dermal partments of low hair follicles into isolated cells and to expose the DPs, at the same time the basal membrane and part ECM of DP were proteolysized. Then repeatedly low-speed centrifugation and panning of the pellet could obtain purely isolated DPs and dermal sheath cells. So firstly, our method takes advantage of the activity of type I collagenase of collagenase D and the activity of type IV collagenase of dispase, through the proteolysis of the membrane and ECM of DP, considerably relieves the labor tension and saves the operated time under stereomicroscope. The time point of digestion is also easy to control through observation under an inverted microscope. When digestion extends to the talk of DP and some DPs are floating in solution, it should be stopped. Secondly, low-speed centrifugation to replace the pickup of the isolated DPs under stereomicroscope can easily separate DPs out from the dermal sheath cells. At the beginning, we picked DPs out from the pellet under stereomicroscope one by one that was also laborious and time-consuming. Afterwards, we improved this procedure by low-speed centrifugation and panning of pellet that was also very effective. Compared with others' results, such as Warren [16], who treated the fleshly DP with collagenase type IV after microdissection, and Chiu [17], who treated the exposed hair bulbs with dispase before microdissection, we obviously state that the operated steps and our method is very easy, more efficient isolation for DPs. Our method remarkably increases DP yield and purity. Thirdly, the method can isolate DP on a large scale, which is very helpful for establishing a DP's cell line, because of the aggregative behavior of DP cells, some DP cells might fail to grow before confluence. Fourthly, the method considerably increases the attack rate and improve cell growth. Finally, our method can obtain the dermal sheath cells at the same time.

We find that the isolated DPs are still intact, showing orbed or ellipsoid shape. They are distinct from digestion with dispase [17, 18], in which method the DPs are partially disrupted by a change in their shape and partial loss of their peripheral definition, because dispase has the activity of fibronectinase and type IV collagenase, but collagenase D mainly has the activity of type I collagenase. So under control of right digestive time, DP will not be disrupted, which can increase the rate of appreciable quantities of primary DP cells per DP. DPs attacked to dermal sheaths cannot be digested first because collagenase D cannot enter the vessel of dermal sheathes due to their vessel being too small.

As others have described the dispase and collagenase type IV treatment [16, 17], the isolated DPs with collagenase D also show significant acceleration of cell outgrowth and enhanced frequency of explant outgrowth and the number of viable cells compared with untreated DPs. Most of them attached within three day and their cells spread out quickly at the same time, which is dif-

Table 1 Results of histological and immunohistochemical staining of DP cells, dermal sheath cells and dermal fibroblasts

Cell type	AB	AB-PAS	Toluidine blue	Vimentin	Desmin	FactorVIII-RA
DP cells	+	++	+	+	−	±
Dermal sheath cells	+	+−	−	+	−	−
Fibroblasts	−	−−	−	+	−	−

AB-PAS is the result of AB and PAS respectively

ferent from the DPs by microdissection that have an attached period for 5–10 days and a stationary phase about 1-3 weeks [7, 14]. The first reason is that the collagenase treatment may expose or activate some structures of DPs that greatly facilitate the attachment of DP explants. In our experiments, the free-floating DP isolated by microdissection could not adhere closely to the wall is the main reason for poorly attacked DP explant, which is probably related to the specific gravity of DP similar to medium and to the intact basal membrane; because when covered with a nylon membrane, DP cells can migrate out. The second reason is that the DP cells migrate out more freely after the basal membrane and part ECM of DP, which might have some function in holding the DP cells together within a structure, are proteolysized. This is supported by the doubling time of DP cells in our study, i.e. only 2.5 days (data not shown).

The ECM of DP consists largely of basement membrane components rather than the interstitial components of the dermis such as collagen types I and III, and it contains laminin, collagen type IV and the basement membrane components associated with chondroitin sulfate including chondroitin 6-sulfate and heparan sulfate proteoglycan, but no chondroitin 4-sulfate [16, 21, 24]. We also find similar results observed in situ DP by others [27, 28], that the cultured DP cells are positive with AB and metachromatical with toluidine blue O. Since positive staining with alcian blue takes place not only at pH 2.5 but also at pH 0.5. It can be concluded that the ground substance of DP cells contains not only nonsulfated acid mucopolysaccharides (AMPS), such as hyaluronic acid, but also sulfated AMPS, such as chondroitin sulfate. Toluidine blue O stain is used for demonstration of metachromasion. At pH 3.0 to 6.0, tissue in hyaluronic acid will show metachromasia. At pH 1.5 and blow, only strong acidic compounds, such sulfated AMPS, will give metachromasia, so DP in situ has a changed-color reaction [29]. Our result shows that the cultured DP cells display changed-color reaction is in line with DP in situ. Furthermore, the combination of AB-PAS counterstained with picric acid is a very colorful and informative stain for demonstration of acid and neutral mucopolysaccharides. We find that the cultured DP cells and the ECM rich in aggregates and clumps of multi-layer DP cells showed strong positive with AB-PAS, which implies that the specific ECM assemble is closely related to the aggregative DP cells. Collectively, the specific expression of ECM within cultured DP cells supports the concept of the DP cells as highly specialized fibroblasts specifically involved in hair growth regulation. These histochemical stain results show that DP cells can synthesize and excrete acid and neutral proteglycan, which indicates the papilla cells in vitro culture in DMEM medium still retain the ability to continue to synthesize and excrete the ECM of DP for a long time, which is consistent with Warren's results [16] and supports the view of the aggregative behavior of papilla cells related to the specific proteoglycan.

Compared with DP cells, the dermal sheath cells can be stained positive with AB and toluidine blue O, but negative with changed-color reaction of toluidine blue O and PAS as same in situ dermal sheath, which suggests that dermal sheath cells also synthesize AMPS in vitro culture condition and have a very close relation with DP cells. Whether the AMPS synthesized by dermal sheath cells is different from the AMPS by DP cells should be further studied.

The DP mainly consists of fibroblasts and endothelial cells. Our results are consistent with this view. We show that a few of DP cells expressed factor VIII-associated protein, which is the endothelial cell mark. It is different from Messenger's [14] and Warren's [16] reports. They all failed to detect factor VIII-associated protein in cultured DP cells. We consider that after treatment with collagenase type I, the endothelial cells in DP are also viable and can migrate out from DP explants. In the same culture condition, they may co-exist with DP cells for some time.

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