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Original article

Optimal subculture methods for passaging and growing human epidermal keratinocytes

Yuanyuan Wang, Caimao Zhang, Tao Yang, Guihong Yang, Shuqian Tang, Jinjin Wu

Department of Dermatology, Third affiliated hospital of Third Military Medical University, Research Institute of wound healing and tissue engineering of Chongqing, Chongqing 400042, China

Background: Human epidermal keratinocytes (HEKs) cover the outer layer of the skin and play a key role in wound repair. Although the methods for isolation and cultivation of primary HEKs from epidermis have been used successfully in both laboratory and clinical settings, the ability to subculture (passage) these cells has yet to be established and is the primary factor hindering their usage.

Objectives: We conducted this study to identify optimal subculture conditions for HEKs.

Methods: We first harvested the primary HEKs from prepuce tissue specimens, and then compared three different reagent compositions (0.25% trypsin, 0.25% trypsin plus 0.01% EDTA, and 0.25% dispase digestion solution) for various periods of time at 4°C with the conventional 0.25% trypsin or 0.25% trypsin plus 0.01% EDTA digestion at room temperature.

Results: Our data indicated that the cold digestion conditions yielded higher cell numbers and more viable cells than the conventional methods. Furthermore, the subcultured HEKs also adhered and grew better after four hours of a 0.25% trypsin cold digestion or after six hours of a 0.25% dispase cold digestion. These procedures produced higher numbers of HEK passages than that commonly seen experienced with conventional methods.

Conclusion: The data from the current study demonstrated that the optimal subculture condition for passaging and growing HEKs *in vitro* is four hours digestion with 0.25% trypsin.

Keywords: *Cell subculture, cold digestion, culture conditions, HEKs, human epidermal keratinocytes*

Human keratinocytes, originating from the ectoderm during embryonic development, cover the outer layer of the skin as a stratified squamous epithelium. This outer layer (known as the epidermis) acts as the body's major barrier against an inhospitable environment. The normal proliferation and differentiation of keratinocytes sustain the structural and physiological function of the epidermis. During the process of skin repair, keratinocytes proliferate and migrate to cover the wounded area and then differentiate and complete the process of re-epidermalization. Since Rheinwald *et al*. succeeded in continuous cultivation of human epidermal cells *in vitro* in 1975 [1], the cultivation techniques used have significantly advanced, allowing keratinocytes to become widely used in studies of skin physiology, pathology, and drug toxicology [2]. Most importantly, human epidermal keratinocytes (HEKs) are now one of the important seed cells for skin re-engineering and wound repair; thus, the *in vitro* cultivation is an essential step towards obtaining HEKs for research and clinical usage.

Based upon morphology and mechanisms of cellular differentiation, the epidermis can be divided into four distinct layers, stratum basale, stratum spinosum, stratum granulosum, and stratum corneum. With the exception of the stratum basale, the cells in all other layers commit to terminal differentiation and become displaced outwards through the epidermal layers. As such, the HEKs from these three layers have low proliferative capacities and displayed poor adhesion ability *in vitro*. Therefore, it is not an easy task to cultivate these HEKs *in vitro* since they demand precise conditions that support their viability

Correspondence to: Jinjin Wu, Department of Dermatology, Third affiliated hospital of Third Military Medical University, Research Institute of wound healing and tissue engineering of Chongqing, Chongqing 400042, China. E-mail: jjw023@yahoo.com.cn

and adhesion ability [3]. In addition, they are more difficult to sustain amongst passaging in a serum-free medium. In our previous study, we demonstrated that the conventional digestion methods using 0.25% trypsin or 0.25% trypsin plus 0.01% ethylenediaminetetraacetate (EDTA) often resulted in incomplete detachment of primary HEKs from the culture dishes. Moreover, we found that the subsequent cells in subculture adhered poorly to the dishes. We also determined that the integrity of the first passage of subculture is particularly important for the long-term survival of HEKs *in vitro* [4]. Thus, the key to successful subculture of HEKs is to choose the optimal time and temperature of enzyme digestion required to achieve complete detachment of the cells while maintaining their biological activity. In this study, we performed a comparative evaluation of different subculture conditions that were theorized to enhance passaging integrity and growth of HEKs *in vitro*.

Materials and methods

Reagents and equipment

Keratinocyte serum-free medium (K-SFM) was obtained from Invitrogen (Carlsbad, USA). Type I collagen was purchased from Sigma (St. Louis, USA), and disapse was from Roche (Basel, Switzerland). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin were supplied by Gibco (Milan, Italy). CASY -Technology was from Innovatis (Bielefeld, Germany).

Specimen acquisition

Prepuce tissue specimens were obtained from children who underwent circumcision in our department, as described previously [5]. This study was approved by the local ethnics committee of Chongqing and all the parents of donors provided written informed consent.

Isolation and culture of primary HEKs

Subcutaneous fatty cells were carefully removed from prepuce, and the resultant prepuce tissues were washed once with 75% ethanol for three minutes, and then three times with phosphate buffered saline (PBS) for five minutes. The tissues were then sliced into pieces of approximately 5mm x 5mm, transferred into 0.25% dispase solution, and incubated overnight at 4°C. On the next day, the epidermis was peeled away from the dermis using a pair of forceps, and the epidermis was digested with 0.25% trypsin at 37°C for 30 minutes. Afterwards, the digestion was terminated with a serum-containing medium and the digestion products were filtered through a sheet of 200-sieve mesh, poured into a 15 ml centrifuge tube, and centrifuged at 1000 rpm for five minutes. Supernatants were discarded and the pellets were washed three times with PBS. The collected cells were mixed with K-SFM medium and re-suspended into single cell suspension. Cell number and viability were determined using CASY®-Technology. Subsequently, the cells were seeded into 24-well culture plates ($5x10^4$ per well) that had been pre-coated with Type I collagen ($5.3\ g/cm^2$ for 10 minutes at room temperature) [6]. The cells were then cultured in an atmosphere of 5% CO_2 and 95% air at 37°C and the culture medium was replaced every 24 hours.

Keratinocyte subculture

Cells were passaged when they reached 70% to 80% confluence. The culture medium was removed from the plates and the culture dish was washed three times with PBS. The following methods were performed for comparative evaluation:

Method 1 (conventional subculture digestions): 0.25% trypsin or 0.25% trypsin plus 0.01% EDTA was added to the culture plates and three wells for each treatment at room temperature. After the cells morphed into a round shape, they were mechanically detached from the dish via gentle tapping on the dish wall. The digestion reaction was immediately terminated by addition of 10% FBS-containing DMEM. The cell suspension was then poured into a 15 ml centrifuge tube by washing the culture dishes twice with PBS and centrifuging the suspended samples at 1000 rpm for five minutes. After that, the supernatants were removed and the cells were then re-suspended in 2 ml K-SFM. Cell viability and numbers were determined using CASY®-Technology. Afterwards, the cells were inoculated ($5x10^4$ per well) into 24-well culture plates pre-coated with Type I collagen. Twenty-four hours later, the culture medium corresponding to each treatment procedure was removed and collected, the cells in the medium were counted by using CASY®-Technology and considered as deciduous cells. The adherence rate of the subcultured HEKs was calculated using the formula: [(number of seeded cells – number of deciduous cells) / total seeded cells] x100%.

Method 2 (cold subculture digestions): 0.25% trypsin, 0.25% trypsin plus 0.01% EDTA, or 0.25%

dispase solution was added, respectively, into the culture plates and three wells for each treatment. Culture dishes were then placed into a refrigerator set at 4°C. At 2, 4, 6, 8, 10, and 12 hours, the culture dishes were removed from the cold and cells were detached from the dish by gentle tapping. After that, the cells were collected by centrifugation at 1000 rpm for five minutes; the remainder of the procedure was identical to that described in Method 1.

Statistical analysis

All values were summarized as mean standard deviation (SD). The SPSS statistical software package version 11.0 (Chicago, USA) was used to assess variance by performance of the Tukey's test. $P < 0.05$ was considered statistically significant.

Results

In the current study, we explored and compared the shedding time, cell number and viability, cell adherence rate after 24 hours inoculation, and number of passages obtained from different subculture methods to determine the optimal conditions for passaging and growing HEKs *in vitro*. First, we compared different time points in which to carry out the cold digestions and found that HEKs were completely released from the dish at two hours after 0.25% trypsin plus 0.01% EDTA treatment; whereas, four and six hours of incubation were required, respectively, for digestion with 0.25% trypsin or 0.25% dispase as can be seen in **Figure 1**.

Next, we compared the cell number and viability after passage using different methods. As shown in **Figure 2**, the conventional methods of 0.25% trypsin or 0.25% trypsin plus 0.01% EDTA generated fewer cell numbers and cell viability than did the three cold digestion methods. In general, the cell viability obtained with the conventional methods was below 80% and the number of viable cells was less than $1x10^5$ per ml. In contrast, the cell viability obtained with the three cold digestion methods was over 80% and the numbers of viable cells were much higher than $1x10^5$ per ml. Furthermore, the conventional 0.25% trypsin and 0.25% trypsin plus 0.01% EDTA digestions resulted in lower adherence rates; the cells adhered and grew much better after 0.25% trypsin cold digestion for four hours than after 0.25% dispase cold digestion for six hours (**Figures 3** and **4**). However, the two conventional digestion methods were only able to support subculture of HEKs for two or three passages, while four hours of 0.25% trypsin and six hours of 0.25% dispase cold digestion supported subculture of HEKs for six to eight passages (**Figure 5**).

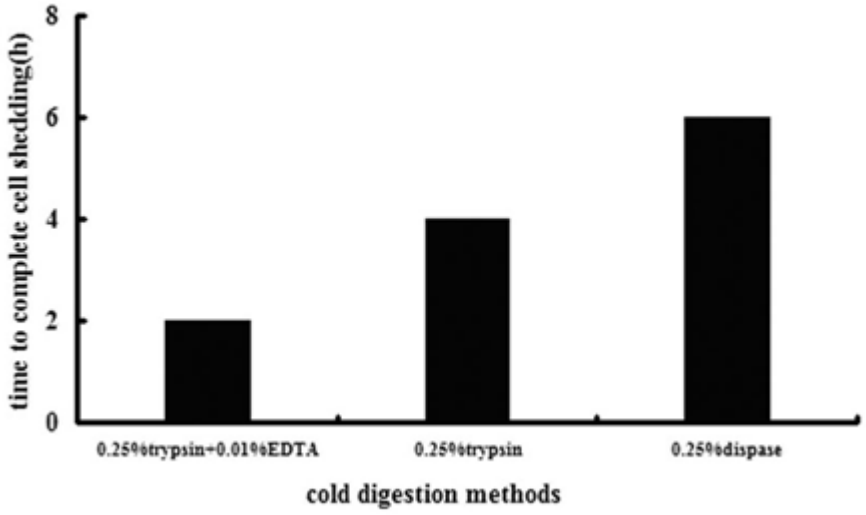

Figure 1. Time to achieve complete cell detachment by the three cold digestion methods. HEKs were detached with cold 0.25% trypsin plus 0.01% EDTA, 0.25% trypsin, or 0.25% dispase digestion solution for 2, 4, 6, 8, 10, and 12 hours, respectively. At each time point, we determined whether the HEKs had yet completely detached from the culture dish. The shortest time for complete cell shedding is presented for each method.

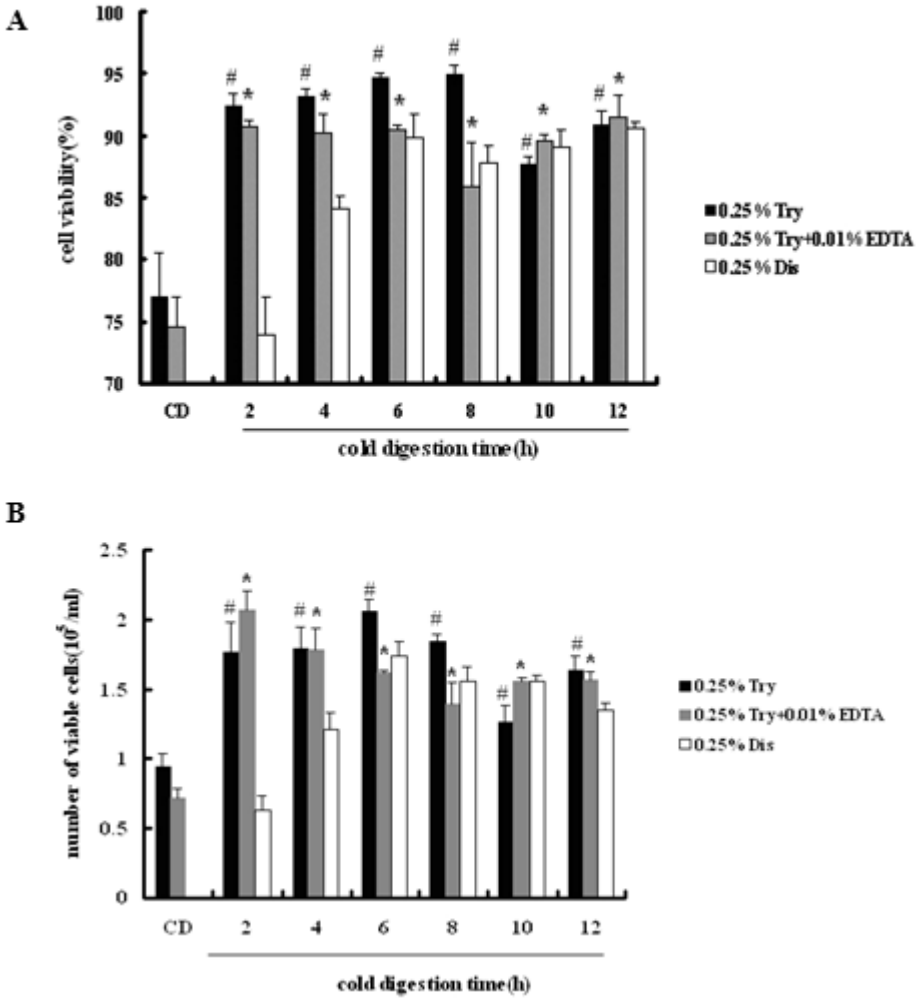

Figure 2. Comparison of cell viability and number of viable cells obtained with conventional digestions (CD) and cold digestions for different time points. HEKs were detached with conventional digestion or cold digestion between 2-12 hours. The cell viability and number were measured by the CASY®-Technology. Data are presented as mean±SD based on three experiments with triplicate dishes. #P <0.05 compared to the 0.25% trypsin (Try) CD. *P <0.05 compared to the 0.25% Try plus 0.01% EDTA CD. Dis, dispase digestion solution. **A**: percent of cell viability, **B**: number of viable cells

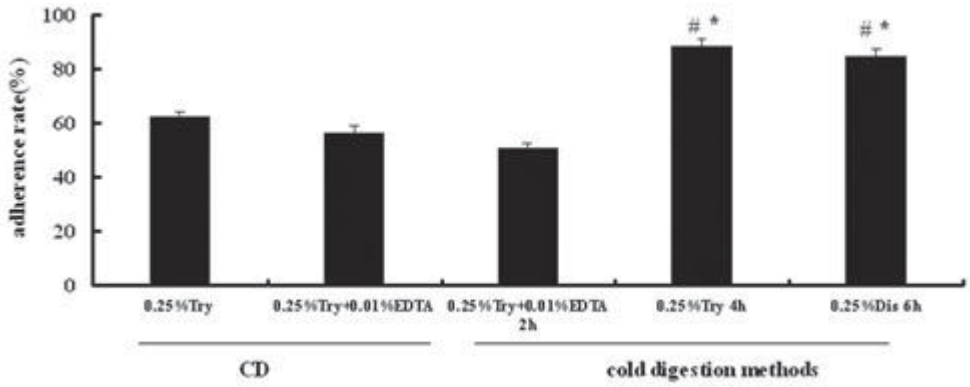

Figure 3. Comparison of the cell adhesion rates between conventional digestions (CD) and cold digestions. HEKs were detached with conventional digestion using either 0.25% trypsin (Try) or 0.25% Try plus 0.01% EDTA, or cold digestion with 0.25% Try plus 0.01% EDTA for two hours, 0.25% Try for four hours, or 0.25% dispase (Dis) solution for six hours. Twenty-four hours later, the cell culture medium was collected and the HEKs in the medium were quantified to calculate the cell adhesive rate. Data are presented as mean±SD based on three experiments performed in triplicate. #P <0.05 compared to the 0.25% Try CD. *P <0.05 compared to 0.25% Try plus 0.01% EDTA CD.

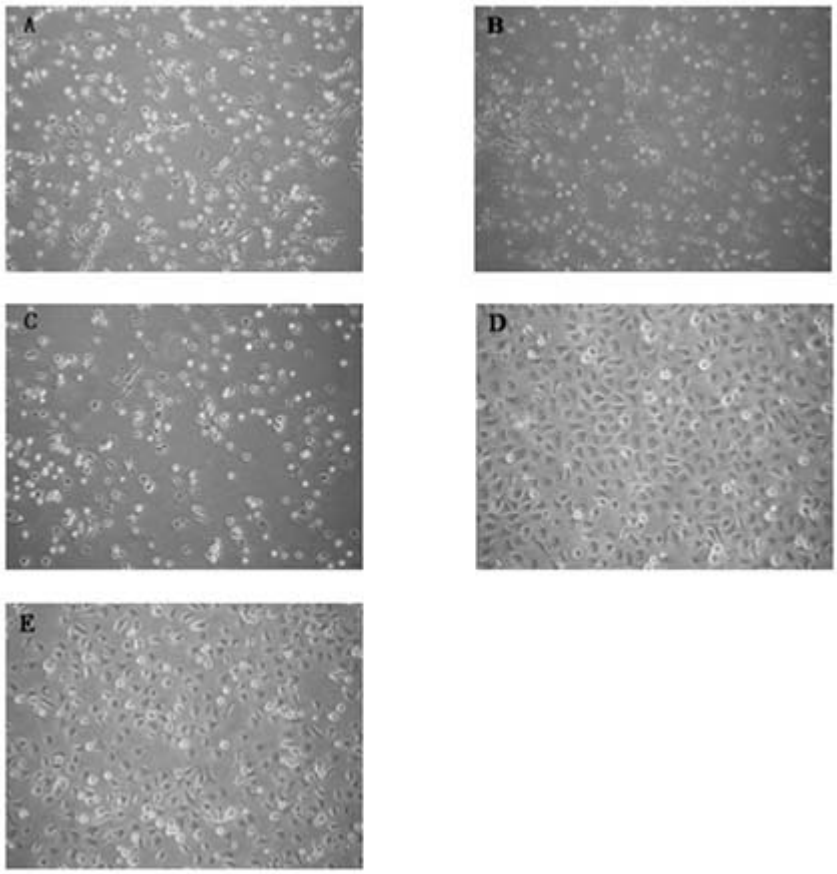

Figure 4. HEK morphology *in vitro*. HEKs were detached with (**A**) conventional digestion with 0.25% trypsin, (**B**) conventional digestion with 0.25% trypsin plus 0.01% EDTA, (**C**) cold digestion with 0.25% trypsin plus 0.01% EDTA for two hours, (**D**) cold digestion with 0.25% trypsin for four hours, or (**E**) cold digestion with 0.25% dispase solution for six hours. Cell morphology was evaluated by inverted phase contrast microscope at 24 hour post-inoculation. Magnification at x 100.

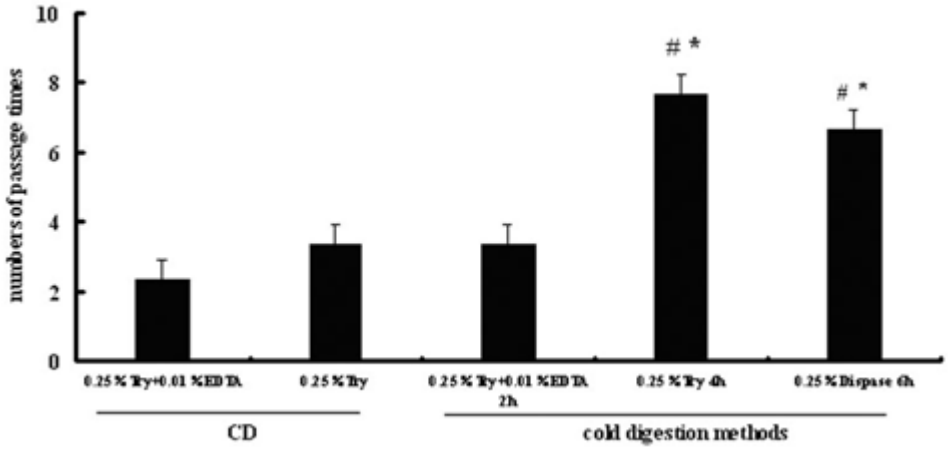

Figure 5. Comparison of HEKs passage numbers attained with conventional digestions (CD) and cold digestions. Primary HEKs and cells from each subsequent passage were detached by means of conventional digestion with 0.25% trypsin (Try) or 0.25% Try plus 0.01% EDTA, or cold digestion with 0.25% Try plus 0.01% EDTA for two hours, 0.25% Try for four hours, or 0.25% dispase (Dis) solution for six hours. The HEKs were then cultivated until they reached approximately 70%-80% confluency when they were harvested and subjected to next subculture; this procedure was repeated until complete loss of the cells occurred. The numbers of passages from each method were recorded and compared. Data are presented as mean SD based on three experiments performed in triplicate. $^{\#}P < 0.05$ compared to the 0.25% Try CD. $^{*}P < 0.05$ compared to the 0.25% Try plus 0.01% EDTA CD.

Discussion

In the current study, we sought to determine the optimal conditions that supported subculture of HEK cells. We prepared excised human prepuce skin samples by first separating the epidermal and dermal layers after incubation with 0.25% dispase solution. We then isolated the primary HEKs from the epidermal layer by using 0.25% trypsin. In order to evaluate the best conditions for subculture of HEKs, we explored three different detachment reagent compositions at 4°C for different points of time. We found that the cold digestion solutions between four and six hours produced higher cell numbers and viability *in vitro* than did the conventional methods. In addition, the subcultured HEKs adhered and grew better after four hours of a 0.25% trypsin cold digestion or after six hours of a 0.25% dispase cold digestion. These procedures also obtained higher numbers of passages than did the conventional methods. The data from the current study provides the optimal subculture procedure for passaging and growing HEKs *in vitro*.

Generally, the growth cycle of HEKs can be divided into the lag, proliferative, and stationary phases, like all other cell cultures [7]. If passaged during the early proliferative phase, HEKs have been shown to be fragile and to experience slow growth. When HEK cells are in the late proliferative phase, they adhere firmly to the culture plate; at this point, if the digestion time is not sufficient, detachment of HEKs from the dish will be incomplete and only a small number of HEKs will be obtained from the subculture. Nevertheless, if the digestion time is too long, the cell membranes of HEKs may be damaged and cell viability will decrease. In the study described herein, we tested various methods to detach HEKs by evaluating different time points. We found that cold digestion solutions used between four and six hours were the most effective at retaining cell number and viability, as well as for their ability to increase the numbers of HEKs passages.

Trypsin is the most commonly used digestive enzyme to achieve cell subculture. For HEK subculture, 0.25% trypsin solution is routinely used [8]. Trypsin hydrolyzes intercellular proteins, thereby dispersing cells and forcing their detachment from the surface of the culture dishes. In conventional subculture, HEKs are treated with 0.25% trypsin solution for a short period of time at room temperature; unfortunately, this procedure causes incomplete detachment of HEKs from the dishes and results in less numbers of cells and viable cells. However, under the cold digestion condition, HEKs could be released completely from the dish surface after four hours, and the cell numbers reached $1x10^5$ per ml and cell viability was well above 90%. In addition, the subcultured cells adhered and grew better, and some HEKs showed a typical cobblestone pattern of growth after 24 hours. However, it is important to remember that prolonged use of trypsin has been shown as able to damage the cell membrane and affect cell viability.

Thus, 0.01% of EDTA is usually added to the trypsin solution in order to maintain the keratinocyte subculture. EDTA acts to chelate Ca^{2+} and Mg^{2+} ions and EDTA is resistant to neutralization with serum. Hence, it is important that EDTA should be removed by centrifugation after digestion of the cell cultures to prevent it from further promoting the release of cultured cells in the subsequent passage. In our hands, 0.25% trypsin plus 0.01% EDTA digestion of HEKs for 2-12 hours showed slow growth with partial adherence. However, there was no significant difference in the numbers of HEK passages that were achieved as compared to the conventional methods.

In addition, dispase acts specifically on hemidesmosomes of the basal membrane and selectively hydrolyzes fibronectin and collagen [9, 10]; thus, dispase can preserve cell viability and intercellular connections. Although dispase may be used to separate the epidermis and dermis from the basal membrane, digestion with this enzymatic reagent should be carried out at 4°C for a longer period of time. For HEKs subculture, the cold dispase solution was applied to the cell culture for at least for 6 h. Due to the preservation of intercellular connections, HEKs treated with dispase solution were always observed to fall off the dish as clumped cells, but cell number and cell viability were lower than that obtained with trypsin solutions. Taken together, our data from the current study showed that HEKs were able to detach from the culture dishes completely after two hours of digestion with 0.25% trypsin plus 0.01% EDTA at 4°C, but the subsequent cell adherence rate was not good and the cells grow poorly. Furthermore, when digestion of the HEK cultures was carried out with 0.25% trypsin or 0.25% dispase for four hours or six hours, much better results were obtained in terms of cell numbers, cell viability, growth rates, and the numbers of passages. In summary, the optimal subculture

condition for passaging and growing HEKs was determined to be four hours digestion with 0.25% trypsin.

Acknowledgments

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