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Original Article

Primary cell culture from human oral tissue: gingival keratinocytes, gingival fibroblasts and periodontal ligament fibroblasts

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Abstract

Primary cell culture of human oral tissue has many applications for oral biology research. There are two techniques in primary culture, which includes the enzymatic and direct explant technique. The objectives of this study were (1) to isolate and investigate the difference in percentage the success in culturing three cell types from human oral tissue: gingival keratinocytes, gingival fibroblasts and periodontal ligament fibroblasts by using the direct explant technique; (2) to compare the effect of sex and age on the success of tissue culturing. Twenty seven tissue samples were obtained from healthy human gingival tissue, 19 female and 8 male patients aged 14-67 years (37.7±17.5). The tissue was cut into 1x1 mm pieces and placed on plastic culture plates containing Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% amphotericin B. For the keratinocytes culture, after the epithelial cells started to multiply around the gingival origin and the diameter was 2-5 mm., the fibroblasts were liminated by mechanical removal under inverted microscope to prevent fibroblast overgrowth and the medium was changed to keratinocyte-SFM (Gibco, BRL) supplemented with 5 µg/ml gentamycin. The results revealed that gingival fibroblast gave the highest success rate in culture (96.3%), followed by gingival keratinocytes (88.9%) and periodontal ligament fibroblasts (81.5%). There was no significant difference in the success rate of cultivation between younger and older individuals, as between sex of the subjects ($p>0.05$). The risk of failure in culture techniques is mainly caused by microbiological contamination from the tissue samples.

Keywords: primary cell culture, direct explant technique, gingival keratinocytes, gingival fibroblasts, periodontal ligament fibroblasts

1. Introduction

The techniques of cell culturing are used to study the gene expression and cytotoxicity testing. Currently, primary cell culturing of human oral tissue has many applications for oral biology research, including the study of differentiation processes, effects of drugs, and chromosomal analysis (Reid *et al.*, 1997). Two techniques that have been used to cultivate oral tissue are the enzymatic and direct explant techniques (Bernice, 1994; Kedjarune *et al.*, 2001; Klingbeil *et al.*, 2009).

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The enzymatic technique was described by Daniels *et al.* (1996), where they surveyed the success rate of human keratinocyte isolation with various concentrations including trypsin and dispase, the enzymatic condition, as well as the calcium concentration in the culture medium. For the keratinocytes yield, the direct explant technique revealed higher proliferation than the enzymatic technique. The operating procedure used in the direct explant technique process involves fewer steps compared with the enzymatic technique (Klingbeil *et al.*, 2009). The direct explant technique has also been used for 30 years in the culturing of human gingival (Lauer *et al.*, 1991) and buccal tissues (Flaxman *et al.*, 1967) and appeared to be more successful than the enzymatic technique in culturing human oral keratinocytes (Kedjarune

et al., 2001). The explant technique combined with autogenous serum can be used for culturing gingival autografts as well as for cultures with special tissues (Lauer *et al.*, 1991). In addition, the direct explant technique obtained the first keratinocytes yield faster than the enzymatic technique (Klingbeil *et al.*, 2009). There have been no studies comparing the direct explant technique for cultivation in primary cell culture of oral tissues. The aims of this study were (1) to isolate and investigate the difference in percentages of the success in culturing three cell types from human oral tissue: gingival keratinocytes, gingival fibroblasts and periodontal ligament fibroblasts by using the direct explant technique; and (2) to compare the effect of sex and age of those who provided tissue samples for the successful tissue cultures.

2. Materials and Methods

2.1 Collection of tissue samples

Gingival tissue for primary gingival keratinocytes (PGK), primary gingival fibroblasts (PGF), and premolar teeth for primary periodontal ligament fibroblast (PPDLF) cultures were obtained from 27 healthy human subjects (age ranging from 14 to 67 years), who were patients undergoing crown lengthening surgery at the Dental Hospital, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The gingival tissue and premolar teeth were placed in Dulbecco's Modified Eagle's Medium (DMEM: Gibco BRL, New York, USA) pH 7.2

2.2 Cell culture

2.2.1 PGK and PGF

The tissue specimens were washed and disinfected in povidone iodine solution for 2-3 min and then washed in culture media (Mlinek *et al.*, 1975; Freshney, 1994; Formanek *et al.*, 1996 and Kedjarune *et al.*, 2001). Gingival tissue specimens were cut into pieces, approximately 1x1 mm in size, and placed in the culture plate (diameter 4 cm) (Corning, New York, USA). The tissue was left in the culture plate for 5-10 min and then the culture plate was flooded with DMEM pH 7.2 supplemented with 10% heat-inactivated foetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 1% amphotericin B (Gibco BRL, New York, USA) to prevent growth of micro-organisms, as culture medium. The culture plate was incubated at 37°C in a humidified atmosphere of 95% air and 5%CO_2 (Incubator HERA Cell 240, Germany) and the old medium was replaced with fresh medium twice a week. After the epithelial cells, which were squamous in shape, started to multiply around the gingival origin to a diameter of 2-5 mm, the fibroblasts were eliminated and removed by a mechanical scraper under an inverted microscope to prevent fibroblast overgrowth, and the medium was changed to keratinocyte-SFM (Gibco BRL, New York, USA) supplemented with 5 mg/ml gentamycin (Gibco BRL, New York, USA). The morphology of PGF resulted in spindle shaped cells. When the primary cell culture reached confluence at 70-80% (the cell number was $1x10^6$ cells/ml), the PGK and the PGF were detached with 0.025% trypsin-EDTA (Life Technology, USA), and 0.05% trypsin-EDTA respectively, diluted with culture medium and then subcultured in a ratio of 1:4. The cells were counted by Coulter Counter Model Z1 (Coulter Electronic Ltd., New York. USA.). PGK was used from the third passages through the fifth passages and PGF was used from the third passages through eighth passages in a T- 75 cm^2 flask (Corning, New York, USA). The success rate in culturing was defined in this study as the ability of the cells to grow out from the gingival origin and become fully explanted, and to survive at least until the first subculture.

2.2.2 PPDLF

The teeth were placed in a petri dish containing DMEM (Lekic *et al.*, 1998). The periodontal ligament was dissected with a sterile scapel blade from the mid-third of the root surface and placed in a culture plate (diameter 4 cm). The tissue was left in the culture plate for 5-10 min and then the culture plate was flooded with DMEM and supplemented with 20% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% amphotericin B, as culture medium. The culture plate was kept in a humid incubator (at 37°C, with 5%CO_2) and the old medium was replaced with fresh medium twice a week. After PPDLF-derived cells (which were spindle-shaped) migrated out of the tissue, the medium was changed to include DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% amphotericin B, as the next culture medium. When the PPDLF confluence was near 70-80% (the cell number was $1x10^6$ cells/ml), it was detached with 0.05% trypsin-EDTA. PPDLF was used from passages 3 through 5 in a T- 75 cm^2 flask. The split ratio used for subculture and the success rate in culturing of PPDLF were the same as for PGK culture.

2.3 Ethical consideration

Informed consent was obtained from the ethics committee of the Faculty of Dentistry, Prince of Songkla University, Hat Yai, Songkhla, Thailand. (Approval # ST/0521.1.03/480; 2008).

2.4 Statistical methods

The Fisher's Exact test was used to determine the difference in the outcome of the three types of cell cultivation by sex of the subjects who provided the tissue samples. A Mann-Whitney *U*-test was used to investigate differences in the three cell types that started to outgrow from the explants with age of the subjects who provided the tissue samples.

3. Results and Discussion

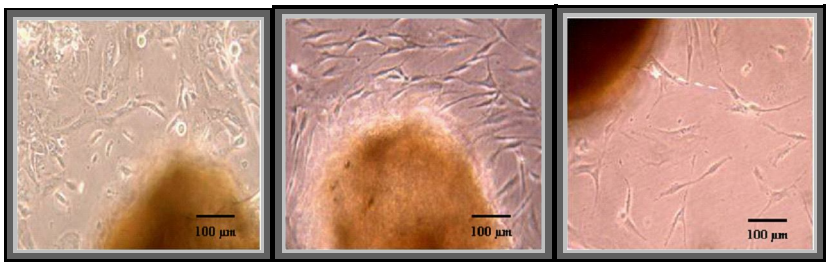
Tissue samples of a size approximately 1x1 mm were

found to grow faster than those tissues larger than 1x1 mm. The direct explant technique required only a small size of tissue samples (Kedjarune *et al.*, 2001). PGF can grow out (3-4 days) faster than PGK (5-7 days) and PPDLF (8-10 days). In comparison, the average initial time for keratinocytes cultivation by the direct explant technique was 14.2 days (Klingbeil *et al.*, 2009) and the epithelial cells multiplied out from the tissue origin after 2-3 days (Kedjarune *et al.*, 2001).

After the three cell types reached 70-80% confluence (the cell number was $1x10^6$ cells/ml), they increased in number. The best possible concentration was 0.05% trypsin-EDTA used for 5-7 min in PGF and PPDLF subculture; whereas 0.025% trypsin-EDTA for 4-5 min was best for PGK, because when using 0.05% trypsin-EDTA in PGK more than 30% of the cells would die in the first passage. Moreover, when the confluence (the cell number was $1x\ 10^4$ cells/ml) of the cell number for PGK subculture was 40-50%, it was found that each subculture growth rate slowed down, which was also described by Reid *et al.* (1997). As this study was adjusted for PGK subculturing, the first passage was sub-cultured to a T-25 flask and then moved to a T-75 flask for the next passage (passage 2-5). This was more successful in increasing the cell number such that the subculture PGF and PPDLF remained in a T-75 flask in the first passage until the tenth passage. In the past, the fibroblasts could be passaged 10-12 times and they were more responsive to trypsin-EDTA than keratinocytes. Therefore, they were easily detached from the culture flasks (Reid *et al.*, 1997).

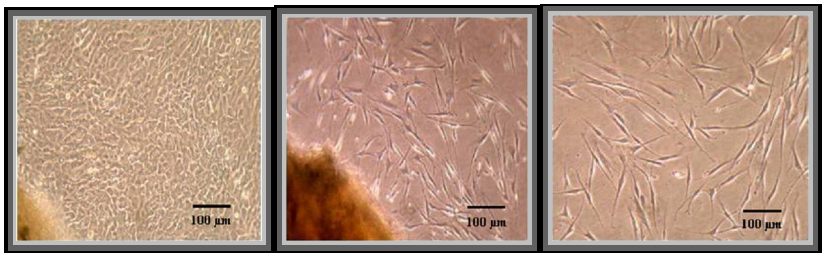
The results revealed that PGF and PPDLF gave the maximum success rate for cell recovering (95%), when compared with PGK (90%), where recovery of the three cell types used equalled $1x10^7$ cells/ml. The cell number was determined with a Coulter Counter (Coulter Z1 New York USA). Three primary cell cultures were examined under an inverted microscope for three different morphologies. The difference in the three cell types of morphology can be observed in Figure 1. After 10 days, the morphology of PGF was spindle in shape, which was the same as PPDLF, while PGK was squamous in shape (Figure 2). Figure 3 shows the three different cells in the first passage at 80% confluence.

Table 1 shows success and failure on direct explant technique classified by sex. Tissue samples were obtained from healthy human subjects between 14-67 years (37.7± 17.5). The total number of tissue samples was obtained from 27 healthy human subjects: 19 female and 8 male patients. The results show that PGF gave the highest success rate in culture (96.3%), followed by PGK (88.9%) and PPDLF (81.5%). In this study the success rate of PGK culturing (88.9%) is higher than in the study of Kedjarune *et al.* (2001) which was about 82% and also higher than the PGK cultures by Reid *et al.* (1997), which had about 80% success even though these two studies used the same direct explant



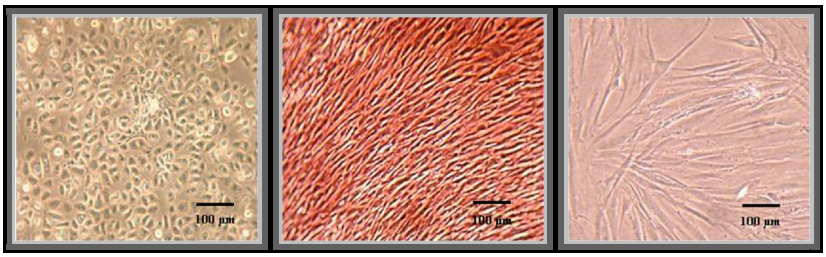
(a) (b) (c)

Figure 1. The morphology of cultured cells, growing from the tissue origin (lower right corner and Upper left corner). (a) Primary gingival keratinocytes. (b) Primary gingival fibroblasts. (c) Primary periodontal ligament fibroblasts.



(a) (b) (c)

Figure 2. The morphology of cells, growing from tissue origin after 10 days of culturing. (a) Primary gingival keratinocytes. (b) Primary gingival fibroblasts. (c) Primary periodontal ligament fibroblasts.



(a) (b) (c)

Figure 3. Three different cells in the first passage at 80% of confluence. (a) Primary gingival keratinocytes. (b) Primary gingival fibroblasts. (c) Primary periodontal ligament fibroblasts.

Table 1. Number, sex and age of the subjects providing the tissue samples on the success in culturing.

Parameter	Direct explant technique					
	Success [a]			Fail [b]		
	Female	Male	Total	Female	Male	Total
Gingival fibroblasts	18	8	26(96.3%)	1	-	1(3.7%)
Gingival keratinocytes	17	7	24(88.9%)	2	1	3(11.1%)
Periodontal ligament fibroblasts	15	7	22(81.5%)	4	1	5(18.5%)
Age (Mean ± SD)	33.1±17.7			41.7±19.5		

[a] The success in culturing means cells can grow around the tissue samples or survival after the first passage.
[b] The cells cannot grow around the tissue samples or survive after the first passage.

technique. In addition, the success of PGF in this study was also higher than in Reid *et al.* (1997), where the success rate was only 63% (Reid *et al.*, 1997). There was no significant difference in the success rate of cultivation between younger and older individuals, as between sex of the subjects ($p > 0.05$). Sex and age of the subjects had no effect on the success rate, which was similar to that described elsewhere (Kedjarune *et al.*, 2001). The mean age for tissue sample success rate was 33 years (33.1±17.7) and 41 years (41.7± 19.5) for tissue sample failure. Previously, 40 years was the age limit for culturing keatinocytes. When tissue samples were taken from subjects who were younger than 40 years of age, the cultured success rate was 80% compared with only 65% for those over 40 years (Reid *et al.*, 1997).

However, isolation and cell cultures in this study remained a problem. For example, there was bacterial contamination in the oral cavity from the tissue samples, which was the same result as Kedjarune *et al.* (2001), Secondly, bacterial contamination was also found during medium preparation, especially the initial step of PPDLF cultivation was easily contaminated from microorganisms, since the tissue samples were very small and thin. Thus, in this study the tissue samples were not disinfected with povidone iodine solution. The optimal times of disinfection for the PGK and PGF were incubation for 5-7 min, which contrasts with other reports (Reid *et al.*, 1997).

4. Conclusion

Culturing of all three cell types with the direct explant technique was easy, rapid, and involved fewer steps. In addition, this technique gave three pure primary cell cultures after the first passage. The sex and age of the tissue samples did not affect the success rate in culturing the three cell types. The direct explant technique can be used in research involved with cell cultures and can also be isolated from other tissues of the body.

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