Video Article

Three-dimensional Inflammatory Human Tissue Equivalents of Gingiva

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Abstract

Periodontal diseases (such as gingivitis and periodontitis) are the leading causes of tooth loss in adults. Inflammation in gingiva is the fundamental physiopathology of periodontal diseases. Current experimental models of periodontal diseases have been established in various types of animals. However, the physiopathology of animal models is different from that of humans, making it difficult to analyze cellular and molecular mechanisms and evaluate new medicines for periodontal diseases. Here, we present a detailed protocol for reconstructing human inflammatory tissue equivalents of gingiva (iGTE) *in vitro*. We first build human tissue equivalents of gingiva (GTE) by utilizing two types of human cells, including human gingival fibroblasts (HGF) and human skin epidermal keratinocytes (HaCaT), under three-dimensional conditions. We create a wound model by using a tissue puncher to punch a hole in the GTE. Next, human THP-1 monocytes mixed with collagen gel are injected into the hole in the GTE. By adimistration of 10 ng/mL phorbol 12-myristate 13-acetate (PMA) for 72 h, THP-1 cells differentiated into macrophages to form inflammatory foci in GTE (iGTE) (IGTE also can be stumilated with 2 μg/mL of lipopolysaccharides (LPS) for 48 h to initiate inflammation). IGTE is the first *in vitro* model of inflammatory gingiva using human cells with a three-dimensional architecture. IGTE reflects major pathological changes (immunocytes activition, intracellular interactions among fibryoblasts, epithelial cells, monocytes and macrophages) in periodontal diseases. GTE, wounded GTE, and iGTE can be used as versatile tools to study wound healing, tissue regeneration, inflammation, cell-cell interaction, and screen potential medicines for periodontal diseases.

Video Link

The video component of this article can be found at https://www.jove.com/video/57157/

Introduction

Periodontal diseases are the leading cause of tooth loss in adults. Gingivitis and periodontitis are the most common periodontal diseases. Both present biofilm-mediated acute or chronic inflammatory changes in gingiva. Gingivitis is characterized by acute inflammation, whereas periodontitis usually presents as chronic inflammation. On the histological level,bacterial components trigger the activation of immune cells, such as macrophages, lymphocytes, plasma cells, and mast cells[1,2]. These immune cells, especially macrophages, interact with local cells (including gingival epithelial cells, fibroblasts, endothelial cells, and osteoblasts) resulting in inflammatory lesions in periodontal tissue[3,4]. Experimental models of periodontal diseases have been established in various types of animals, such as rats, hamsters, rabbits, ferrets, canines, and primates. However, the physiopathology of animal models is different from that of humans, making it difficult to analyze cellular and molecular mechanisms and evaluate new medicines of periodontal diseases[5]. Co-cultivation of periodontal bacteria and monolayer human oral epithelial cells has been used to investigate the mechanism of periodontal infections[6]. Nevertheless, monolayer cultures of oral cells lack the three-dimensional (3D) cellular architecture of intact tissue; therefore, they cannot mimic the *in vitro* situation.

Here, 3D inflammatory human tissue equivalents of gingiva (iGTE) are established to represent periodontal diseases *in vitro*. This 3D model of periodontal diseases occupies an intermediate position between monolayer cell cultures and animal models. Three types of human cells, including HaCaT keratinocytes, gingival fibroblasts, and THP-1 macrophages, are co-cultivated on collagen gel, and stimulated by inflammatory initiators to build iGTE. IGTE closely simulates the *in vivo* conditions of cell differentiation, cell-cell interaction, and macrophage activation in gingiva. This model has many possible applications for drug screening and testing new pharmacological approaches in periodontal diseases, as well as for analyzing cellular and molecular mechanisms in wound healing, inflammation, and tissue regeneration.

Protocol

This protocol is designed to create human gingival tissue equivalents, gingival wound models, and gingivitis models. Human skin epidermal keratinocytes (HaCaT) were kindly provided from Professor Norbert E. Fusenig of Deutsches Krebsforschungszentrum (Heidelberg, Germany)[7]. Human gingival fibroblasts (HGFs) were isolated from gingival tissues according to the previously published protocols[8]. Informed consent was

obtained beforehand, and the study was approved according to the guidelines set by the Committee of Ethics, the Nippon Dental University School of Life Dentistry at Tokyo (Authorization Number: NDU-T2012-35). Protocol steps 1–3 should be performed in a cell culture hood.

1. Preparation of 3D Human Tissue Equivalents of Gingiva (GTE) (Figure 1A)

1. Mix collagen type I-A gel with 10% concentrated MEM-alpha and 10% reconstruction buffer (2.2 g of $NaHCO_3$ and 4.47 g HEPES in 100 mL 0.05 N NaOH) in a 15 mL sterile tube by pipetting. Keep the mixed collagen solution on ice until used.
2. Place 24-well culture inserts (pore size 3.0 µm) into a 24-well plate.
3. **Remove HGFs from the culture surface using 0.25% trypsin-EDTA solution. Suspend the cells in 10 mL of culture medium A (MEM-alpha + 20% FBS + 1% GlutaMAX). Count the cells by a Bürker-Türk cell counter.**
    1. Extract the desired quantity of cells (1-5 x10^5 cells/mL), then centrifuge for 4 min at 190 x g.
    2. Suspend the cells in the mixed collagen solution. Keep the mixture on ice until the next step.
4. Add 0.5 mL of the cell-collagen mixture (0.5–2.5 x 10^5 cells/well) into the culture insert without producing any bubbles. Incubate the mixture for 10–30 min in a humidified atmosphere with 5% CO_2 at 37 °C to coagulate the mixture into a gel.
   NOTE: Add the collagen mixture into the center of the culture insert to avoid unequal formation of collagen gel.
5. Add 1 mL of the culture medium into the well and 0.5 mL into the insert. Incubate the culture plate for 24 h or overnight in a humidified atmosphere with 5% CO_2 at 37 °C.
6. Remove HaCaT cells from the culture surface using 0.25% trypsin-EDTA solution. Suspend the cells in 10 mL of medium B (DMEM + 10% FBS + 1% GlutaMAX) and count the cells. Extract the needed quantity of cells (1–5 x 10^5 cells/mL) with a pipette, centrifuge for 4 min at 190 x g. Suspend the cells in medium B.
7. Remove the medium from the culture inserts, which contain the HGF-collagen mixture, by aspiration. Add 0.5 mL of HaCaT cell suspension (0.5–2.5 x 10^5 cells/well) on the top of the HGF-collagen mixture. Incubate the cultures for 24 h or overnight.
   NOTE: At this time point, the medium in the culture well should be medium A, while the medium in the culture insert should be medium B.
8. Replace the culture medium with KSR medium (co-culture medium) (MEM-alpha + 15% KnockOut Serum Replacement + 1% GlutaMAX) + 5% FBS. Add 1 mL of the culture medium into the culture well and 0.5 mL into the culture insert. Incubate the cultures for 24–48 h.
9. Replace the culture medium with KSR medium + 1% FBS. Add 1 mL into the culture well and 0.5 mL into the culture insert. Incubate the cultures for 24 h or overnight.
10. Replace the culture medium in the culture well with 0.7–1 mL KSR medium. Remove the medium completely from the culture insert (the culture surface should be exposed to air). Incubate the cultures for 1–2 weeks. Change the medium in the culture well two times per week.
    NOTE: Do not let the medium level rise above the culture surface (the top layer composed of keratinocytes). Always keep the culture surface dry.

2. Preparation of Wounded GTE (Figure 1B)

1. Prepare a 1–3 mm diameter tissue puncher. Sterilize it with an autoclave at 121 °C for 20 min.
2. Push the tissue puncher perpendicularly into GTE about 300 µm deep, and punch a hole in the tissue to simulate a wound.
3. At this step, use the wounded GTE for investigating wound healing and tissue regeneration. Alternatively, fix GTE using 10% formalin neutral buffer solution to perform histological analysis.

3. Preparation of Inflammatory GTE (iGTE) (Figure 1B)

1. Cultivate THP-1 cells according to the previous report[9].
2. Mix collagen type I-A gel with 10% concentrated RPMI 1640, 10% reconstruction buffer (2.2 g of $NaHCO_3$ and 4.47 g HEPES in 100 mL 0.05 N NaOH) and 10 ng/mL PMA. Keep the mixed collagen solution on ice until use.
3. Count 1–2 x 10^6 THP-1 cells and centrifuge for 4 min at 190 x g. Suspend the cells in 1 mL of the mixed collagen solution. Keep the mixture on ice until the next step.
4. Add 10–30 µL THP-1-collagen mixture into the wounded area of GTE. Replace the culture medium to 0.7–1 mL KSR medium containing 10 ng/mL of PMA.
5. Incubate the mixture in a humidified atmosphere with 5% CO_2 at 37 °C for 72 h.
6. Use the model for investigating gingivitis or periodontal disease (for example, add potential anti-inflammatory medicine to iGTE to see its effects on cytokine release[10]). Alternatively, omit 72 h PMA-treatment, add 2 µg/mL of LPS into the culture, and incubate the tissue in a humidified atmosphere with 5% CO_2 at 37 °C for 48 h[11,12].

4. Fixation and Whole Mount Immunostaining of GTE and iGTE Cultures

1. **Fixation of the cultures.**
    1. Remove the culture medium from the 24-well plate. Wash the tissues 2 times with phosphate buffered saline (PBS).
    2. Add 1 mL of 10% formalin neutral buffer solution to the insert and 1 mL to the culture well. Leave the 24-well plate in a refrigerator at 4 °C overnight.
    3. Remove the 10% formalin neutral buffer solution the following day.
    4. For whole mount immunostaining, wash the tissues 3-4 times with PBS after fixation.
    5. For hematoxylin and eosin (H&E) staining and regular immunostaining, proceed with dehydration, paraffin embedding, and sectioning.
       NOTE: For whole mount immunostaining, this step can be omitted.
2. **Whole mount immunostaining**
   NOTE: This protocol was modified from the one in reference 13.
    1. Wash the tissues 3x in PBS 0.5–1% Triton, 10–30 min each time.

2. Incubate the tissues twice for 30 min in blocking buffer (PBS 1% Triton + 10% BSA), at room temperature.
3. Wash the tissues 2× in blocking buffer, 5–10 min each time.
4. Add 50 µL of primary antibody solution at the required dilution/concentration to the inserts. Use a thin plastic cling film to wrap the inserts to avoid drying out the tissue.
5. Incubate the tissue for 1 to 2 days at 4 °C.
6. Wash the tissues 3 times, for 30 min in PBS 1% Triton + 10% BSA. Wash again 3 times, for 5 min in PBS 1% Triton.
7. Add 50 µL of secondary antibody in blocking buffer (PBS 1% Triton + 10% BSA) and 5 µL of Hoechst 33342 solution (NucBlue Live Cell stain) to each insert. Use a thin plastic cling film to wrap the inserts to avoid drying out the tissue.
8. Incubate for 1 to 2 days at 4 °C. Wrap the 24-well plate in a wet paper towel, and then put it into a plastic pack to be used throughout incubation.
9. Wash the tissues 3 times, for 10 min in PBS 1% triton
10. Use a sharp needle to cut the membrane of the culture insert to obtain the tissue. Place the membrane onto a slide.
    NOTE: The tissue should be on the membrane.
11. Add 2–3 drops of fluorescence mount medium. Cover the tissue with a cover glass and store at 4 °C in the dark until analysis.
12. View tissues with a confocal laser scanning microscopy (LSM).

Representative Results

HaCaT cells displayed typical keratinocyte morphology under phase-contrast microscopic observation (**Figure 2A**). Scanner electron microscopic (SEM) images showed that HaCaT cell surfaces were covered by many microvilli. Intercellular connections between HaCaT cells were mediated by membrane processes (**Figure 2B**). HaCaT cells expressed gingival epithelium marker K8/18[14], indicating that HaCaT cells are suitable for gingiva reconstruction (**Figure 2C**).

Figure 3A shows a successful example of GTE, and **Figure 3B** shows a failed example of GTE. The unsuccessful trial (**Figure 3B**) is caused by the collagen gel mixture not being added in the center of the culture insert. **Figure 3C** showed that a wound model of GTE can be made by punching a hole in the tissue with a tissue puncher. H&E staining (**Figure 3D**) and SEM images (**Figure 3E**) demonstrated that at 19 days of cultivation, about 10–20 layers of HaCaT cells form epithelium, and collagen gel-embedded HGF cells form lamina propria. In the lamina propria of the reconstructed GTE, HGF cells exhibited characteristic morphology of fibroblasts (**Figure 3F**), and were surrounded by well-defined arrays of collagen fibers (**Figure 3G**). Immunofluorescence staining showed that vimentin and TE-7 (both of them are dermal fibroblast markers[15]) are expressed in the lamina propria of GTE (**Figure 4A** and **4B**). K19, a specific marker to junctional and pocket epithelium in periodontitis[16], is weakly expressed in the epithelium of GTE (**Figure 4C**), and is strongly expressed in the epithelium of iGTE (**Figure 4D**). The data suggested that HaCaT cells can differentiate into key epithelial cells in periodontitis, especially after inflammatory stimulation of the lamina propria.

Inflammatory gingival diseases, such as gingivitis and periodontitis, are histologically characterized by the presence of lymphocytes and macrophages, and result in fibrosis and tissue necrosis[17,18]. In this protocol, THP-1 cells were used as the inflammatory cells for iGTE. As shown in **Figure 5**, without PMA-stimulation, THP-1 cells presented a monocytic morphology and most of them floated in the medium (**Figure 5A**). In contrast, after being exposed to PMA 10 ng/mL for 72 h, more than 50% of THP-1 cells differentiated into macrophage-like cells and adhered onto the culture surface (**Figure 5B**). Similar to HaCaT and HGF cells[8], THP-1 cells grew well inside the collagen gel in the co-culture medium (KSR medium), and differentiated into macrophage-like cells after PMA-treatment (**Figure 5C**). Immunostaining showed that the adhered THP-1 cells expressed CD68 (a marker of macrophages[19]) (**Figure 5D**) and CD14 (another marker of macrophages) (**Figure 6**). To form iGTE, THP-1 monocytes were embedded in the collagen gel and injected into the hole simulating a wound (**Figure 5E**). At 72 h after PMA (10 ng/mL)-stimulation, THP-1 cells differentiated into macrophages (**Figure 5F**) inside the hole simulating a wound and expressed CD68 (**Figure 5G**), indicating they are able to form inflammatory foci in the wounded GTE.

Whole mount immunohistochemistry (**Figure 6**) showed that THP-1 cells in the hole simulating a wound not only expressed CD14 (another marker of macrophages), but also expressed vimentin as well as the fibroblasts in the edge of the hole. Vimentin is expressed by active macrophages[20]. The data confirmed that THP-1 macrophages are functional.

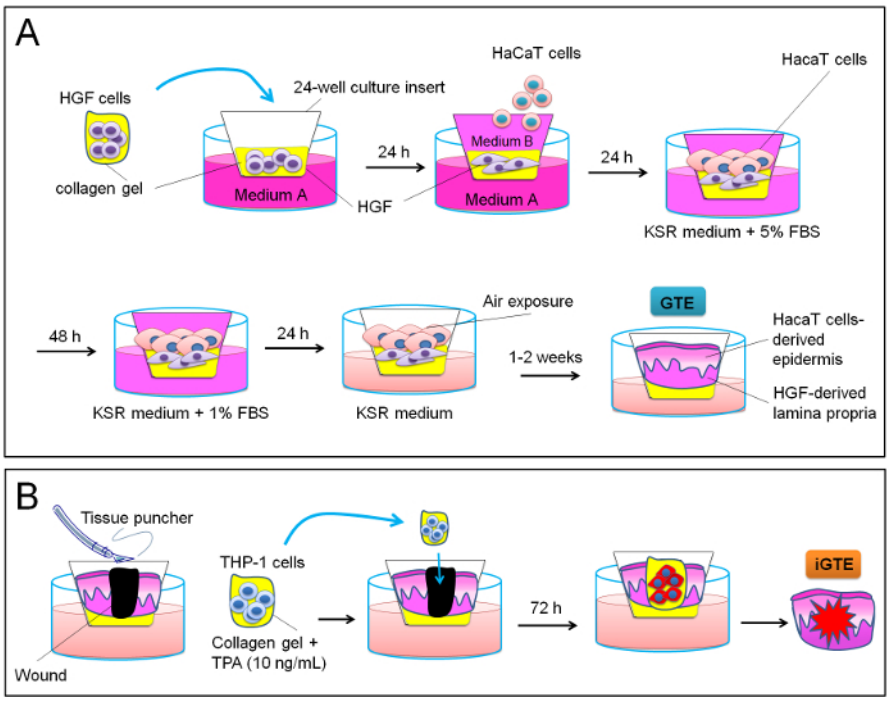

Figure 1: Experimental designs. Experimental setup of 3D human tissue equivalents of gingiva (GTE) (**A**) and inflammatory GTE (iGTE) (**B**). Please click here to view a larger version of this figure.

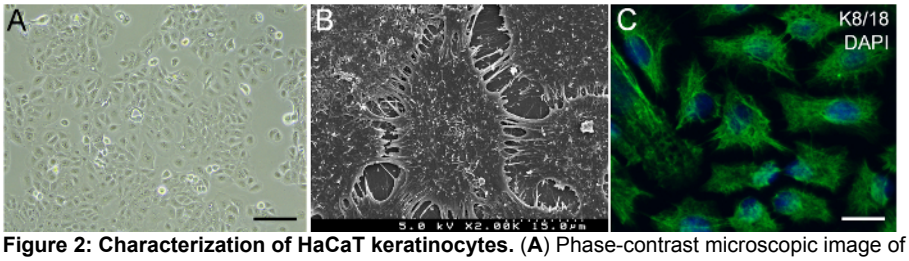

Figure 2: Characterization of HaCaT keratinocytes. (**A**) Phase-contrast microscopic image of HaCaT cells in culture. Scale bar = 50 µm. (**B**) SEM image of HaCaT cells. (**C**) Immunohistochemical staining of HaCaT cells for gingival epithelial cell marker K8/18. Green, K8/18. Blue, DAPI. Scale bar = 10 µm. Please click here to view a larger version of this figure.

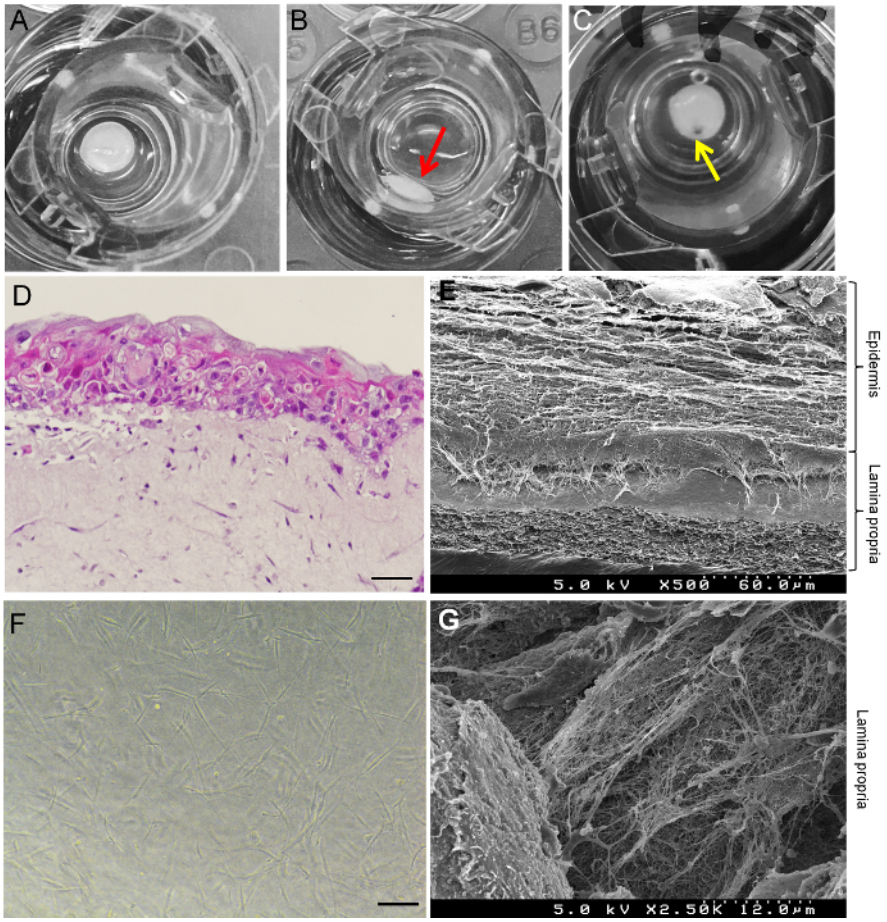

Figure 3: Characterization of 3D human tissue equivalents of gingiva (GTE). (**A**) Example of a successful GTE in culture. The tissue is in the middle of the culture insert. (**B**) Example of a failed GTE: Part of the GTE is separated from the tissue and biased toward the wall (Red arrow). (**C**) Wounded GTE: Yellow arrow indicates a hole punched by the tissue puncher. (**D**) H&E staining of GTE cultivated for 19 days (14 days air exposure). Scale bar = 50 µm. (**E**) SEM image of a vertical section of GTE cultivated for 19 days. (**F**) Phase-contrast microscopic image of HGF cells in collagen gel that formed the lamina propria of GTE. Scale bar = 25 µm. (**G**) SEM image of massive collagen fibers around HGF cells in the lamina propria of GTE. Please click here to view a larger version of this figure.

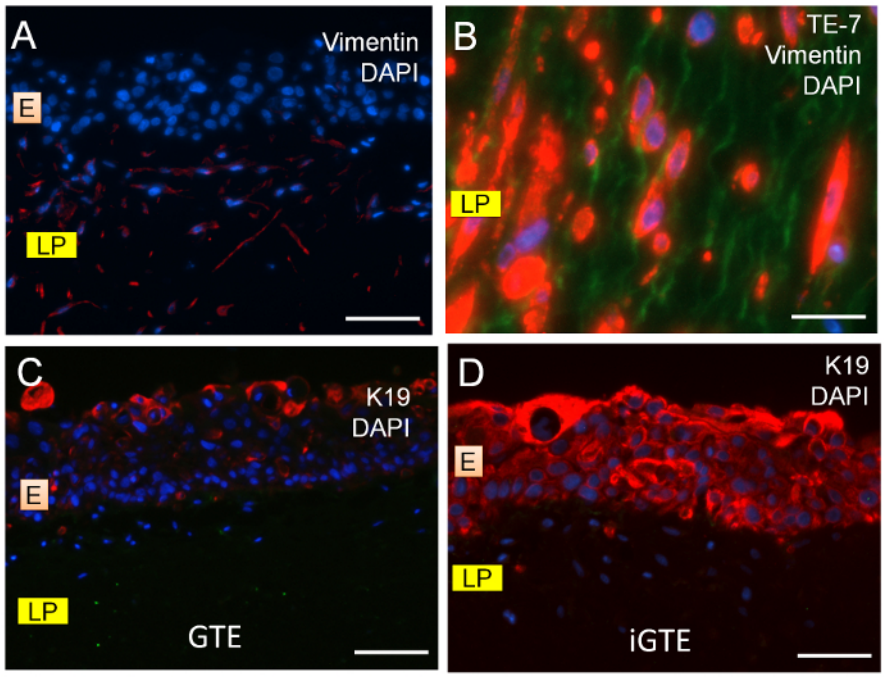

Figure 4: Expression of vimentin, TE-7 and K19 in GTE. Immunofluorescence staining of GTE for lamina propria markers vimentin (**A** and **B**) and TE-7 (**B**) in GTE, and epidermis marker K19 in GTE (**C**) and iGTE (**D**). Red, vimentin and K19. Green, TE-7. Blue, DAPI. E, epidermis; LP, lamina propria. Scale bar = 50 μm (**A**, **C** and **D**), 20 μm (**B**). Please click here to view a larger version of this figure.

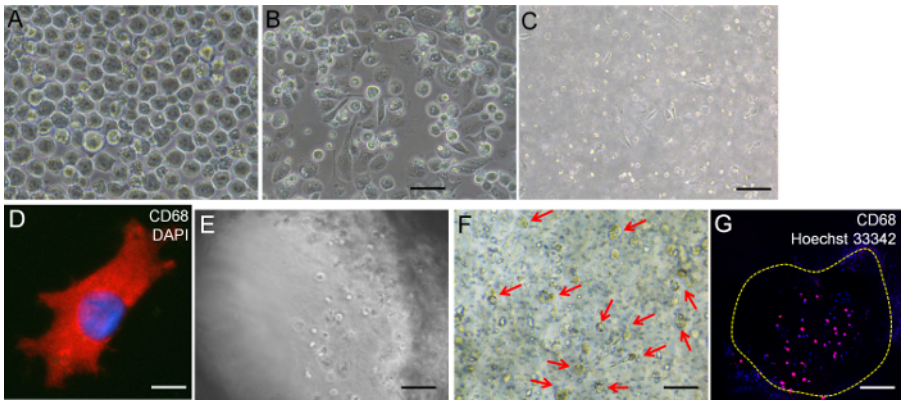

Figure 5: Characterization of THP-1 cells. (**A**) Undifferentiated THP-1 cells in culture. Scale bar = 15 μm. (**B**) Differentiated THP-1 macrophages. Scale bar = 15 μm. (**C**) THP-1 cells were embedded in collagen gel in KSR medium and treated with PMA for 24 h. Scale bar = 50 μm. (**D**) Immunohistochemical staining of THP-1 macrophages for macrophage marker CD68. Red, CD68. Blue, DAPI. Scale bar = 5 μm. (**E**) THP-1-collagen mixture in the wounded hole of HGE at the beginning of PMA treatment. Scale bar = 25 μm. (**F**) THP-1 cells differentiated into macrophages (red arrows) at 72 h after PMA treatment. Scale bar = 25 μm. (**G**) THP-1 cells expressed macrophage marker CD68. Yellow dashed line indicates the area of the wounded hole in H&E. Scale bar = 100 μm. Red, CD68. Blue, hoechst 33342. Please click here to view a larger version of this figure.

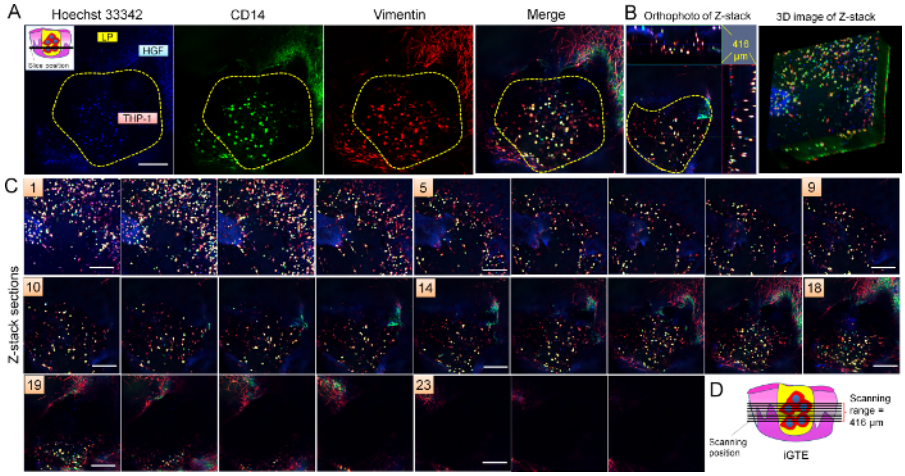

Figure 6: Characterization of iGTE. Whole mount immunohistochemistry for CD14 (green) and vimentin (red) was performed on iGTE. Z-series images (**C**) captured through iGTE (416 µm in total. Panel 1 is the first section LSM scanned, and panel 25 is the last. The interval is about 16.64 µm between sections). Representative section (**A**) (No. 17 of the Z-series images), orthophoto and 3D image of Z-stack (**B**) presented the 3D distribution of CD14- and vimentin- positive THP-1 macrophages in the hole simulating a wound in the iGTE. The scanning position of LSM in iGTE is indicated in (**D**) and the left top corner in (**A**). Scale bar = 50 µm. Yellow dotted lines indicate the area of the wounded hole in iGTE. Please click here to view a larger version of this figure.

Discussion

This protocol is based on methods of creating gingival tissue equivalents and subcutaneous adipose-tissue equivalents described by previous reports[8,21,22]. Although this is a simple and easy method, some steps require special attention. For example, the collagen mixture should be kept on ice until use to avoid gel formation in the solution. When adding the collagen mixture into the culture insert, make sure the solution was injected in the center of the insert to avoid forming a biased gel (like in **Figure 3B**). Punching a hole in GTE also takes skill. The tissue puncher sometimes cannot pick up the tissue if the tissue is too wet. A tissue biopsy system or a pair of forceps would be helpful; however, the dimensions of the wounded location will be much changed. It is also possible to use a syringe with sharp needle to inject the THP-1 cell-collagen mixture without punching a hole (make sure the gel does not clog the needle).

The limitation of this method is that it is unable to perfectly represent the complex biological phenomena of gingivitis and periodontitis, because iGTE lacks blood vessels, neuronal cells, and other immune cells, and the inflammation is not caused by the host-biofilm interactions. However, it is the only *in vitro* gingivitis tissue model made by human cells that partly presents the pathology of inflammatory gingiva. The model can be modified to be more similar to the real gingivitis and periodontal disease by 1) co-cultivating GTE with biofilm to create host-biofilm interactions or stimulating inflammatory responses in iGTE by using LPS (the compounds in the outer membrane of Gram-negative bacteria and a strong inflammatory initiator as we suggested in the protocol), 2) seeding the collagen-HGF solution on extracted teeth to form gingiva-tooth complexes and observing how inflammation in gingiva influences hard tissue, and 3) using human gingival epithelial cells to form the epidermis of GTE and iGTE.

With the methods presented here, GTE and iGTE cultures can be used as versatile tools to study wound healing, tissue regeneration, inflammation, cell-cell interaction, and to screen potential medicines for gingivitis and periodontal disease.

Disclosures

The authors declare no conflict of interest.

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