Quantitative evaluation system for tissue-engineered corneal epithelial cell sheets using image-based technology

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Abstract

Background: Tissue-engineered corneal epithelial cell sheets have been applied for treating ocular surface diseases. It is essential to establish a standardized validation system for fabricated cell sheets before transplantation to spread the therapy in the world. It has already been reported that a validation system for tissue-engineered corneal epithelial cell sheets was developed, in which several sliced frozen sections of sheet were fluorescently stained and the each was subjectively quantitated under a microscope with naked eye. In this study, we attempted a platform for an evaluation system which can be more simplified in preparation steps and more objective in quantification by automatically observing wider area of sample areas.

Methods: Human limbal epithelial cells isolated from corneoscleral rims of cadaveric donors (n=4) were cultured with 3T3 feeder layer cells on a temperature-responsive culture insert for two different culture periods, for day 15 and 28. The fabricated cell sheets, without slicing them, were fluorescently stained with five antibodies and with DAPI for nuclear staining, and then a wider area of the sheets were automatically scanned by a confocal microscope and quantitatively analyzed using a 2-D image processing technique with combination of two spatially-cropped images, lower and upper, which are created by dividing stacked image at the center of its z-direction.

Results: The cell sheets harvested on day 15 consisted of 4-6 layers of cells, the number of which in the lower was about 1.5 to 2.0 times larger, compared to that in the upper while on day 28 they had only 2-3 layers with the decreased number of cells by about 50% in the lower. The differences in the protein expressions the culture periods brought about were successfully quantitated by image processing technique as well. On day 15, ZO-1, AE5 and MUC16 were predominantly expressed in the upper while the expression of panCK was seen in every cell on the sheet. On day 28, the expression levels of ZO-1, AE5, MUC16 and panCK were decreased in the both layers with some unique features. Cells expressing p63 were predominantly observed in the basal layer of the sheets on day 15, but the level was decreased on day 28 as well.

Conclusions: The combinational image-based technology could be one of the powerful tools in establishing a standardized validation system not only for corneal treatment but for some other tissue-engineered ones.

Keywords: Tissue-engineered cell sheets, regenerative medicine, quantitative analysis, standardized system, image processing, confocal microscope

Introduction

Corneal epithelial stem cells are seen in the basal layer of transitional zone called limbus between the cornea and the bulbar conjunctiva [1]. They are responsible for renewal and restoration of the corneal epithelium by generating progeny (transient amplifying cells), which are committed to epithelial differentiation, with the capabilities of migrating into the corneal basal layer from the limbus [2,3]. Corneal opacification with severe visual impairment results from limbal disorder with complete loss of corneal epithelial stem cells caused by some severe trauma or eye diseases, where the peripheral conjunctival epithelium invades inwardly due to the lack of its sources of corneal epithelial cells, with its surface enveloped by vascularized conjunctival scar tissue [4,5]. Limbal allograft transplantation is commonly performed for patients with unilateral or bilateral limbal stem-cell deficiencies [6], which, however, can require long-term immunosuppression that involves high risks of serious eye and systemic complications [7].

Graft failure is often seen in patients with the Stevens-Johnson syndrome or ocular because of pemphigoid serious preoperative conditions such as persistent inflammation of the ocular surface, abnormal epithelial differentiation of the ocular surface, severe dry eyes, and lid-related abnormalities [7,8,9].

Several groups have recently reported that epithelial cell transplantation was really effective treatment for those suffering from these severe ocular surface diseases [10,11,12]. Nishida *et al.*, also showed a regenerative therapy with transplantation of functional tissue-engineered epithelial cell sheets fabricated on a temperature-responsive culture dish, the surface of which is coated with polymers of poly N-isopropylacrylamide (PIPAAm) which reversibly change its property of hydrophobic/hydrophilic depending on temperature across 32°C, where the cultured cells can be easily harvested in a single form of contiguous cell sheet without using proteolytic enzymes like trypsin solution only by reducing temperature below 32°C [13,14].

Hayashi *et al.*, have recently developed a validation system

to confirm whether or not a tissue-engineered epithelial cell sheet is transplantable in terms of the quality, considering that nobody has reported on how to validate final products for transplantation despite the fact that the corneal epithelial regenerative therapy, one of the most advanced applications of regenerative medicine, could be developed into one of the standard medical treatments from clinical trials [15]. Thinly sliced sections of cell sheets fluorescently-stained with four antibodies (not simultaneously but separately), p63 (stem/progenitor cells) [16], AE5 (differentiation), ZO-1 and MUC16 (barrier function) [17,18] were observed under a microscope and subjectively evaluated with naked eye, while cells obtained by dispersing a part of the cell sheets were also stained with anti-panCK antibody (purity of epithelial cells) and quantitatively analyzed by flow cytometry [15].

Methodology with Image-based analysis has been increasingly developed especially to control cell quality for clinical cell therapy [19,20]. Kato *et al.*, developed a non-destructive image-based system for cell quality assessment by applying their own unique algorithms without fluorescently staining cells [21]. Kim *et al.*, developed a novel system to characterize spatial cell distribution in multilayer sheet of human keratinocytes in the proliferative and growth-arrested states using a confocal laser scanning microscope in combination with image processing technology [22].

In this study, we attempted direct observation of the whole of cell sheets stained with the five antibodies (p63, AE5, ZO-1, MUC16 and panCK), without preparing sliced sections of cell sheets, using a confocal laser scanning microscope and quantification with image processing technology what is called high content analysis, which allows quantitative image analyses by extracting information on spatial distribution of fluorescently-labeled components and providing statistical data, in order not only to reduce time-consuming multiple steps of preparing samples but also to fill gaps among researchers in qualitatively evaluating samples under the microscope with naked eye [23,24]. The methodology of automatically scanning samples makes it possible to observe much wider range of fields than the one of observing only a thinly-sliced section, which could provide more detailed and accurate information. We compared cell sheets between two different culture periods, for day 15 and 28 referring to the previous report [15], according to which, cells' density in a dish on day 10 was so low that they found it impossible to detach it as a cell sheet due to the short culture periods. On the other hand, they showed that cell sheets on day 15 (normal culture periods) and day 28 (excess culture periods) could be detached although some sheets on day 28 might be detached partially-broken.

Materials and methods

Epithelial cell culture

Human limbal tissues were isolated from corneoscleral rims of cadaveric donors (Northwest Lions Eye Bank, Seattle, WA).

The tissues were washed with Dulbecco's phosphate-buffered saline (Sigma-Aldrich, Canada, Ontario) containing antibiotics and antibacterials (Life Technologies Inc. Carlsbad, CA), and incubated with dispase II (3 U/ml) (Life Technologies Inc.) at 37°C for 1 hour in order to separate the epithelial layer. The layer was then treated with trypsin-ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) at 37°C for 10min. The cells were plated on temperature-responsive 6-well culture inserts (CellSeed, Tokyo, Japan) in KCM medium (Dulbecco's modified Eagle's medium (DMEM)/F12 [3:1]) supplemented with 10% fetal bovine serum (Japan Bio Serum, Hiroshima, Japan), 0.5% Insulin-Transferrin-Selenium (ITS; Life Technologies Inc.), 2.0×10^{-9} M triiodothyronine (MP Biomedicals, Aurora, OH), 0.4 µg/ml hydrocortisone succinate (Wako, Osaka, Japan), 10 ng/ml EGF (Life Technologies Inc.) and 1.0×10^{-9} M cholera toxin (Wako, Osaka, Japan), at 150,000 cells per insert where NIH/3T3 cells (ECACC, Salisbury, UK) treated with 10 µg/ml of mitomycin C have been prepared on the bottom plates.

Antibodies

In order to make multi-immunofluorescent staining of cell sheet possible, anti-cytokeratin 3/2p mouse monoclonal antibody (AE5; PROGEN, Germany) was fluorescently labeled with Alexa Fluor 488 monoclonal antibody labeling kit (Life Technologies Inc.). Fluorescence conjugated antibodies, anti-ZO-1-Alexa Fluor 594 (mouse monoclonal; 1A12) and anti-pancytokeratin (panCK) -FITC (mouse monoclonal; Ks pan1-8) were purchased from Life Technologies Inc. and PROGEN Biotechnik GmbH (Heidelberg, Germany), respectively. Anti-MUC16 antibody (mouse monoclonal; Ov185; AbCAM, Cambridge, UK) was labeled with Alexa Fluor 647 Zenon mouse IgG labeling kit (Life Technologies Inc) just before staining step. Rabbit anti-p63 polyclonal antibody was purchased from LIFESPAN (Seattle, WA).

Immunofluorescence staining

The fabricated cell sheets with trans-well membrane were cut away with the knife from its insert container along with the curve without detaching cell sheets from the membrane. They were fixed with cold methanol at 4°C for 10 min. and washed with Tris-buffered saline (TBS; Takara Bio, Shiga, Japan). Cell sheet with membrane, which is helpful in cropping images because of its flatness, was bisected for immunofluorescence staining (Figure 1). One of the bisected cell sheets was incubated with group1 solution containing Alexa Fluor 488-conjugated anti-AE5 and Alexa Fluor 594-conjugated anti-ZO-1 antibodies, and the other was incubated with group2; FITC-conjugated anti-panCK, anti-p63 and Alexa Fluor 647-labeled anti-MUC16 antibodies in TBS-based staining buffer containing 5% donkey serum (Sigma-Aldrich) and 0.3% Triton X100 (Wako) for 40 min. at room temperature, and washed twice with TBS-based washing buffer containing 0.05% tween20 (Affymetrix, Santa Clara, CA). The group 2 was additionally incubated with secondary antibody, Alexa Fluor

555 goat anti-rabbit IgG antibody (Life Technologies Inc.) for 20 min. in the staining buffer. The both groups of the sheets were stained with 5 μg/ml of DAPI (Roche, Basel, Schweiz) for 5 min. at room temperature, and then washed with the washing buffer three times and mounted on a slide glass with TBS. For negative controls, samples were also stained with their own isotype controls; Alexa Fluor 488 mouse IgG1 (Biolegend, San Diego, CA), Alexa Fluor 594 mouse IgG1 (Cell Signaling Technology, Danvers, MA), FITC mouse IgG2a (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit IgG (R&D Systems, Minneapolis, MN) and normal mouse IgG1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Confocal laser scanning microscopy

To acquire images of the fluorescently-stained cell sheets, we used the confocal laser scanning microscope FLUOVIEW (FV10i-W; OLYMPUS, Tokyo, Japan), which can scan a wide area of sample with motorized XY-stage automatically through 60X objective lens. Fluorescence of DAPI, Alexa Fluor 488 (or FITC), Alexa Fluor 555 (or Alexa Fluor 594) and Alexa Fluor 647 were detected with its detector of Blue (420–460 nm), Green (490–540 nm), Red (570–670 nm) and Far-red (660–710 nm), respectively. The images from twenty positions per group

of sample, in which four consecutive frames constitute one position of image, were taken at 1.0 μm step in the z-direction (**Figure 1**).

Image cropping and conversion

Figure 1 indicates experimental procedure for Immunostaining and image acquisition to image processing analysis. The acquired four frames of images were tiled to constitute one position of image (XY: 424 x 424 μm) and stacked in the form of three dimensions (XYZ). The 3-D stacked images were then spatially cropped at the center of the Z-direction into two layers of images, "lower" and "upper", with cellSens software (OLYMPUS, Tokyo, Japan), based on the positional information of DAPI-stained nuclei in the following procedure; (1) decide each baseline of the "lower" (1-1) and "upper" (1-2) at the point where no DAPI signal is detected in the image but it should be the closest image from the last DAPI-detectable one (usually 3 to 5 μm distance from the tip of the nucleus), (2) decide the Z-center position of the image by subtracting Z-value of the "upper" baseline" from the "lower" one, (3) crop the "lower" from the lower baseline to the centerline and then the "upper" from the centerline to the uppermost image beyond the upper baseline because some other

Figure 2. Segmentation of fluorescence images by image processing.

(a) Fluorescently stained nuclei of cells were segmented by image processing based on their shapes, morphology and brightness level. Detected nuclei are indicated as colored ones (upper right). (b) ZO-1 expression was detected by “string” module Traced ZO-1 signals are shown as gray lines in the picture (lower right).

signals except nuclear signal can be expressing beyond the upper baseline. The two spatially-cropped 3-D images were then projected separately into 2-D images. The projected images were quantitatively analyzed with CELAVIEW image processing software (RS100; OLYMPUS, Tokyo, Japan) [25,26]. The 3-D images were also reconstructed with Volocity image software (Perkin Elmer, Waltham, MA) to visually check them at different angles.

Quantitative image analysis

The forty projected images including “lower” and “upper” per group of sample were quantitatively analyzed with CELAVIEW software. Total fluorescent intensity per image was used to analyze expression levels of MUC16, AE5 and panCK. The number of the cells and their nuclear area (indicated by pixel) were automatically measured by object segmentation which can make several connecting DAPI-stained nuclei individually separated based on the threshold of their brightness, shapes and morphology (Figure 2a). Mean fluorescence intensity of Alexa Fluor 555-labeled p63 in the segregated nucleus was also quantitated at a single cell level. The expression level of ZO-1 was measured using a module with a unique algorithm, “string”, which is mainly used for detecting long and thin forms of objects such as neurites and angiogenesis tubule formation (Figure 2b).

Results and discussion

2-D Image processing technique with combination of two spatially-cropped images, lower and upper, which are created

Figure 3. Representatives of 3-D and X-Z reconstructed images of cell sheets on day 15 and 28.

Cultured cell sheets were immunofluorescently stained with five antibodies; group1: AE5 (Alexa Fluor 488: green), ZO-1 (Alexa Fluor 594: magenta), group2: MUC16 (Alexa Fluor 647: blue), p63 (Alexa Fluor 555: red) and panCK (FITC: green), and the both groups were counterstained with DAPI (cyan) for nuclei.

* These two images of panCK are seen from the bottom of cell sheets.

by dividing stacked image at the center of its z-direction, successfully quantitated the fabricated epithelial cell sheets without directly analyzing the images of 3-D-reconstructed cell sheets. Figure 3 indicates the 3-D and X-Z reconstructed images of the cultured corneal epithelial cell sheets on day 15 and 28, which were immunofluorescently stained with five antibodies and DAPI for nuclei. The projected images are also shown in Figure 4.

The epithelial cell sheets harvested on day 15 had 4-6 layers of the cells while those on day 28 looked thinner and fragile because of consisting of only 2-3 layers of cells as shown in Figure 3. As indicated in Figure 5, on day 15, the number of the cells counterstained with DAPI in the lower was about 1.5 to 2.0 times larger, compared to that in the upper layer (Figure 5a). It was, however, decreased by about 50% in the lower on day 28 (except sample 4). Corneal differentiated

epithelial cell marker, AE5 (cytokeratin 3/2p), predominantly expressed in the sheet although its expression was very faint in the basal layer of the sheets, which was consistent with the results of quantitative image analysis. The AE5 expression level in the upper layer was about 1.4 to 1.9 times higher than the one in the lower (**Figure 5c**). As to pancytokeratin (panCK), one of the markers indicating purity of epithelial cell, little difference was seen between the lower and upper layer in the expression level although, compared to the upper layer, slight increase in its expression in the lower could be observed because of the larger number of the cells in the lower (**Figure 5d**). Membrane-associated mucin (MUC16), which is specifically seen on ocular surface where it plays an important role in epithelial barrier function, was continuously expressed throughout superficial cells as if it were covering the whole other cells in the lower layer. The MUC16 expression in the upper was over 5 times more than that in the lower (**Figure 5e**). The expression level of tight junction marker (ZO-1) detected with “string module” which recognizes a net-like structure (**Figure 2b**), was about 6-9% in the upper layer of images and below 1% in the lower (**Figure 5f**).

The cells expressing p63, stem/progenitor cells’ marker, were predominantly in the lower layer (**Figure 3**), and over 50% of p63-positive cells in the cell sheet (lower + upper) were seen in the lower while the upper layer had below 25% (**Figure 5g**). To confirm the expression of p63 in the lower and upper layers, the cells were visually shown by “image gallery”, one of the most useful tools of CELAVIEW (**Figure 6**).

On the other hand, in the epithelial cell sheet harvested on day 28, similar tendency could be observed in the five protein expression level in sample1, 2 and 3 although sample 4 had very slight decreases or no changes in the expressions except that of p63 expression (**Figure 5g**). The expression levels of AE5, panCK, ZO-1, MUC16 and p63 were decreased in the both lower and upper layers although these decreasing degrees varied depending on samples. Some round shaped cells with higher fluorescence intensity of AE5 and panCK were mainly seen in the upper layers. The signal of ZO-1 was disconnected and faint in almost all the upper layers (< 2%) as shown in **Figure 3** and **Figure 4**, while the ZO-1 net-like structure was little detected in the lower layer as well as on day 15 (< 1%). The loss of MUC16 signal or its “cave-in” in the surface of the cell sheets on day 28, which sometimes contributes to increase in MUC16 signal in the lower layer, was observed in some parts of the cell sheet where contiguous MUC16 expression must have been seen throughout superficial cells on day 15 although the damaged level of the cell sheets greatly varied among samples.

Additionally, we also analyzed the cell sheets on nuclear area of the cells stained with DAPI by randomly selecting 2000 cells per each layer. On day 15, the average of the nuclear area in the upper (approx. 500 pixels) was 1.5 times larger than that of the lower (approx. 800 pixels) (**Figure 5b**). More interestingly, cells with larger nuclear area were primarily detected in the lower on day 28 (**Figure 4** and **Figure 5g**).

In this study, we analyzed two cropped and projected images, “lower” and “upper”, using the 2-D image processing software to obtain statistical results rapidly, not with stereoscopic methodology which is impractical at present to statistically process massive amounts of 3-D images although it can directly analyze 3-D images. Spatial division of 3-D image at the center of the Z-position into two 2-D images enables us to successfully represent prominent features of the bottom layer and superficial layer in corneal epithelial cell sheets. In addition, all the images were taken with high transparent insert membrane without detaching it so as to hold flatness of bottom layer constant for image cropping and also avoid further damages of cell sheets especially harvested on day 28.

On day 15, we successfully obtained the stable results that all of the four samples showed similar values in each protein expression, the number and the nuclear area of the cells with a certain level of the ratio (lower/upper). More importantly, little differences among 20 positions of the acquired images per group of sample were seen in the parameters (**Figure 7**,

Figure 5. Quantitatively analyzed data on cultured corneal epithelial cell sheets on day 15 and 28. Projected images of “lower” and “upper” layers were quantitatively analyzed with CELAVIEW. White and gray bars show data on day 15 and 28, respectively. (a) the total number of the cells, (b) area per nucleus, (c, d, e) total fluorescence intensity of AE5, panCK and MUC16, and (f) percentage of positive ZO-1 signal in the lower or upper image, (g) percentage of positive p63 signal in the lower plus upper image. Graphs are shown as average $\pm$ SD obtained from 20 positions' measurements. FI: total fluorescent intensity, *; not detectable “control”; representative sample of data stained with isotype control. (No differences among samples were not seen in the isotype controls.)

day 15), which indicates that cell sheets harvested on day 15 consist of stable and uniform cell layers so that they can form a population when shown in scatter plot of the parameters. On day 28, the level of the protein expression in sample 1, 2 and 3 tended to be declined in accordance with the decreased number of the cells although the values varied depending on positions of the sheets so that the scatter plot on day 28 tended to scatter downward (Figure 7, day 28).

As to the sample 4 on day 28, it didn't have any damages

the other three samples had on day 28 in their cell sheets, and the rise in some proteins' expression, far from being the decline, could be seen. We don't know the reason for little or no change in the parameters of the sample 4 because each sample used in this study had different conditions in the elapsed time (from death to surgical removal of eyeball, from removal to the start of stem cell culture) and in the delivery from Seattle in the USA to Tokyo in Japan, to say nothing of the genetic background.

We also demonstrated that analysis of nuclear area of the cells showed outstanding difference in size between the lower and the upper layer. This difference may possibly be another new parameter for evaluating cell sheets. It would be essential to standardize an evaluation system in order to spread some treatments with regenerative medicine into the world, and it could be also accelerated by a rapid, objective and statistical quantification system in combination with interactive function of the software between visual images and its numerical data as indicated by “image gallery” in **Figure 6** [26].

Conclusion

We were successfully able to evaluate the differences in the protein expressions the culture periods brought about by using combination of the confocal laser scanning microscope and image processing technology. The microscope has made it possible to directly observe tissue-engineered corneal epithelial cell sheets without preparing thinly-sliced sections, which requires time-consuming process and skilled technique, not to mention the fact that it can automatically scan a wider range of areas or positions of cell sheets. Observation of multicolor-stained cell sheets allows us not only to save time

and labor but also to get multi-information of parameters from exactly the same sample at the same time. Moreover, objective and statistical quantification of cell sheets by the image processing technique which allows analyzing data under the same conditions, no matter who may do it, can reduce human-error and minimize deviation between individuals, compared to subjective one under the microscope through the eyepieces with naked eye.

Comprehensive combinational study of “quantitative evaluation before transplantation” and “clinical tests’ results after transplantation” would be needed to create a specific and appropriate validation standard system for transplantation of corneal epithelial cell sheets. This image-based technique must be helpful for evaluating some other cell sheets. Kim *et al.*, evaluated early-stage maturation of human retinal pigment epithelial cells to semi-quantitatively analyze the formation of

ZO-1 tight junction and judge the stratification by counting total and overlapping nuclei [27], which might give some hints to quantitatively analyze stratification we did not do with image processing in this study.

All told, quantitative evaluation system based on imaging technique, sometimes providing far more information than expected, would be a powerful tool in establishing a validation standard before transplantation for regenerative medicine although this study is just an example to show how useful combinational image-based technology is.

Competing interests

Tamiyo Kobayashi submitted a patent application entitled "Evaluation method for cells with multi-layer structure using imaging technology (Appl. No.195938) on September 6, 2012.

Authors' contributions

T. Kobayashi was responsible for conception, design, execution, interpretation and preparing the manuscript of this study. M. Yamato and T. Okano provided advice on fabrication methods of corneal epithelial cell sheets, assays, interpretation of results and the manuscript. Y. Watanabe assisted in all aspects of this study mentioned above and interpretation of data. All authors have had the opportunity to provide manuscript revisions and they have approved the final version.

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