The Morphological and Biomechanical Changes of Keratocytes Cultured on Modified p (HEMA-MMA) Hydrogel Studied by AFM

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Summary: The poor integration with host cornea tissue and the low mechanical properties of pHEMA hydrogel for artificial cornea remains a difficult problem to solve. A modified pHEMA hydrogel, MMA copolymerized and type-I collagen and bFGF immobilized, was previously prepared in an attempt to solve the problems. In this study, the cytotoxicity of Col/bFGF-p (HEMA-MMA) and p (HEMA-MMA) was studied by cell adhesion assay and atomic force microscopy (AFM). The results of cell adhesion assay show that the attachment of keratocytes on the modified membrane is much higher than that of the unmodified membrane. This indicates that the material after modification have better cell–material interaction. The AFM images reveal that the morphology of keratocytes cultured on different substrate is obviously different. The cell cultured on modified membrane presented a completely elongated and spindle-shape morphology. The force-distance indicates that the biomechanical of keratocytes changes significantly after culturing on different substrates. The adhesion force (2328 ± 523 pN) and Young’s modulus (0.51 ± 0.125 kPa) of the cell cultured on modified membrane are much higher, and the stiffness (0.08 ± 0.022 mN/m) is lower than those of the cell cultured on unmodified membrane. These results show that the cytotoxicity of Col/bFGF-p (HEMA-MMA) for keratocytes is much improved.

Key words: atomic force microscope (AFM), Col/bFGF-p (HEMA-MMA), morphological structure, ultrastructure, biomechanical properties

Introduction

There are probably more than 10 million people worldwide suffering from blindness of corneal origin, and their visual recovery relies largely on the successful grafting of donor corneal tissue. Indeed, the success rate of this operation is quite high in certain situation. However, the insufficient supply of donor organs and graft immune rejection are remaining limit factors. The only desirable alternative to donor graft transplantation is artificial cornea.

The research of artificial cornea has been lasting for over three hundred years and kinds of artificial cornea have been designed and fabricated. In the late 1950s, Chirila created the first “core-and skirt”, PHEMA hydrogel-based keratoprosthesis, a device known commercially as AlphaCor (Chirila 2001). However, the poor compatibility with host cornea and the low mechanical properties of PHEMA hydrogel frequently lead to the extrusion of artificial cornea (Hicks et al. 2000; Lee et al. 1996; Lou et al. 1999). Therefore, the mechanical strength and surface modification of PHEMA hydrogel to support the adhesion and growth of keratocytes were required.

In the previous work, we copolymerized HEMA with MMA, and then successfully immobilized type-I collagen and bFGF onto the copolymer surface. The primary studies showed that the Col/bFGF-p (HEMA-MMA) possess better mechanical properties and lower cytotoxicity than p (HEMA-MMA) (Yan et al. 2009). In spite of that, whether the Col/bFGF-p (HEMA-MMA) could support the adhesion of keratocytes and its cytotoxicity for keratocytes were still unknown.

Any changes in morphology and biomechanical properties of cells often reflect their health states, and
atomic force microscopy (AFM) is a powerful tool to detect these changes (Cia et al. 2009; Guck et al. 2005; Greenleaf et al. 2007; Pelling et al. 2005; Suresh 2007). Studying these changes in the cell will be helpful to understand the cytotoxicity of materials.

In this study, the keratocytes were cultured on the modified and unmodified p (HEMA-MMA), the cell adhesion assay was carried out first, and the morphology and biomechanical properties of keratocytes cultured on different substrates were characterized by AFM at the single cellular level. By comparing these changes of the cells, the cytotoxicity of the Col/bFGF-p (HEMA-MMA) for keratocytes could be easily speculated.

Materials and Methods

All reagents used in the experiments were of analytical grade. DMEM culture medium was purchased from Beijing Newsprobe Biotech. Co., Ltd (Beijing, China). FBS, penicillin, and streptomycin were obtained from Hangzhou Sijiqing Bioengineering Material Co., Ltd (Hangzhou, China).

Cell Culture

The eyes were obtained from New Zealand white rabbits. The eyeballs were washed with sterilized phosphate buffered saline (PBS) supplemented with penicillin (100 U/ml) and streptomycin (100 U/ml) three times. Next, the corneas were excised with ophthalmic scissors. The excised corneas were washed with PBS. The stroma was separated from the epithelial layer and minced into small pieces with forceps. Then, the stroma piece was placed in a spinner flask containing 0.25% trypsinase. After digested in an incubator (37°C, 5% CO₂) for 3 h, the pieces were centrifuged at 1,000 rpm for 10 min. The cell suspension was cultured at 37°C, 5% CO₂, and 95% humidity in the DMEM medium supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), and 10% FBS. The culture medium was changed every 3 days.

Cell Adhesion Assay

The modified and unmodified p (HEMA-MMA) membranes were placed on the bottom of 24-well tissue culture plates (Costar). Cell adhesion rate was determined at 24 h after a seeding density of 3 × 10⁴ cell/ml on each well and tissue culture polystyrene (TCPS). Before harvesting the adherent cells by trypsinization, twice-gentle washings with PBS were performed. The cells were counted using a haemocytometer. The adhesion rate was defined as the percentage of the cell number counted on the sample to the cell number counted on TCPS.

Cell Preparation for AFM

The keratocytes were cultured on the modified and unmodified p (HEMA-MMA) membrane for 24 h. After washing with PBS twice, the cells were immediately fixed in 2.5% glutaraldehyde for 15 min at room temperature. Then washed and equilibrated in PBS three times, shortly air-dried at room temperature, and immediately imaged and measured in morphology and biomechanical force by AFM.

AFM

AFM (Autoprobe CP Research, Veeco) was performed in a contact mode in air (room temperature; humidity: 75%). The unmodified and modified membranes carrying keratocytes were mounted onto the XY stage of AFM, and an integral video camera was used to locate and relocate the regions of interest. The curvature radius of silicon tips was less than 10 nm. Observation was carried out in air at room temperature. The scan speed of the tip was 0.5–1 Hz. The AFM images were planar leveled using the software (Thermo-microscopes Proscan Image Processing Software Version 2.1).

The contact mode was used for measuring adhesion forces of keratocytes. AFM-based force spectroscopy was used for force detection. Force–distance curves were obtained by standard retraction. All the force–distance curves were measured at the same loading rate.

Results and Discussion

The cell adhesion rates of modified, unmodified, and TCPS membranes are shown in Figure 1. The modified membranes have higher cell adhesion rate (nearly 90%) than the unmodified group (down to 60%). It indicates that the modified membrane could support the adhesion of keratocytes and is appropriate for artificial cornea materials.

AFM Analysis of Morphology of Keratocytes Cultured on Different Substrate

An organism’s each cell has its specific size and shape, which has a specific function. The morphology
structure has great influence on the function of physiological and pathological processes. Previous studies revealed that cell morphology will affect the adhesion, proliferation, and differentiation (Addae-Mensah and Wikswo 2008; Schmitz and Gottschalk 2008). Once the cells cannot maintain the inherent shape, its function would be jeopardized, which would bring a range of issues (Binnig et al. 1986).

With this in mind, we investigated the morphology of two keratocytes cultured on unmodified and modified substrate using AFM (Figs. 2 and 3). Figure 2 shows the morphology of keratocyte cultured on unmodified membrane, the cell represents a partially spread, atrophy appearance (Fig. 2(A, B)), with a height of 3.5–4.0 μm and a width of 25–30 μm (Fig. 2(F)). For the cell in Figure 3, AFM reveals a classical spread out, polygonal appearance cell (Fig. 3(A, B)), with a height of 1.1–1.3 μm and a width of 45–55 μm (Fig. 3(F)). The keratocyte in Figure 3 spreads fully, completely elongated, and presents spindle-shape morphology. It indicates that the modified membrane shows better cell–material interactions than the unmodified.
Comparison of Ultrastructure of Keratocytes Cultured on Different Substrate

The high-resolution advantages of AFM make it a suitable tool for studying cell ultrastructures (Zhao et al. 2003; Chen et al. 2003; Hu et al. 2008), so we compared the ultrastructure of keratocytes cultured on different substrates next. As it can be seen from Figures 2(C–E) and 3(C–E), the ultrastructures of the two keratocytes are quite different. The ultrastructure images show clear grains on two cell surfaces. Then, we analyze the ultrastructures of the two cells (Figs. 2(C) and 3(C)) respectively by using software IP 2.1.

Figure 4 shows that the grain diameters of two keratocytes are accorded with normal distribution. The ultrastructure grain sizes of cell cultured on modified membrane (Fig. 4(B)) are relatively bigger, ranging in size between 23.3 and 209 nm, and mainly distributed around 140 nm, whereas the grain sizes of cell cultured on unmodified membrane ranging between 7.50 and 67.7 nm, mainly around 50 nm. Furthermore, four parameters (Ave Rough, Arm Rough, Mean Height, and Median Height) of the ultrastructure are obtained by the software IP 2.1. As we can see from Figure 5, four parameters of keratocytes cultured on modified membrane are higher. It indicates that the cell membrane are much rougher, the grains are bigger and denser than that of cell cultured on unmodified membrane.

As we know, the diameter of cell membrane protein is generally about 20–30 nm. However, the grain diameters of both the two keratocytes (Fig. 4) are far beyond the scope. This phenomenon could attribute to the metabolism of the cell. When the cell was alive, the cell membrane protein adsorbed the nutrients, such as lipids and carbohydrate, to maintain the process of cell metabolism. This implies that the larger its grain diameter, the more nutrients absorbed, and the more exuberant its metabolism.
AFM Force Curve Analysis of Biomechanical Properties

Biomechanics has a very important role in the metabolism process of cell. Cell growth, proliferation, migration, adhesion, and differentiation are highly dependent on and regulated by cellular mechanical properties (Laurent et al. 2005; Nagayama et al. 2001; Puecha et al. 2006). Therefore, a thorough understanding of cell mechanical is very useful to recognize the state of cell.

To compare the difference in biomechanical properties of cell, the adhesion force was measured by acquiring force–distance curves. Figure 6 presents representative force–distance curves. From the force curves, the value of the adhesion force, relative stiffness, and Young's modulus can be obtained as follows.

As can be seen from Figure 6, the adhesion force \( F \) can be obtained from the distance of the bottom to the equilibrium line.

The stiffness can be obtained from the equation:

\[
\text{Stiffness} = \frac{Y(\text{pN})}{X(\text{nm})}
\]  \hspace{1cm} (1)

That is, the slope of the retraction curve is the stiffness of cell (Fig. 6).

Young's modulus calculations, based on the basic Hertz model (Brochu and Vermette 2008; Dimitriadis et al. 2002), were done on samples of keratocytes cultured on modified and unmodified membranes, to be able to compare the impact of these states on the calculated modulus. These models show the relationship between the adhesion force \( F \) and the Young's modulus \( E \), as shown in the following equation:

\[
F = \frac{4}{3} \frac{ER^{1/2} \delta^{3/2}}{(1 - v^2)}
\]  \hspace{1cm} (2)

Young's modulus can be calculated as

\[
E = \frac{3}{4} \frac{1 - v^2}{R^{1/2} \delta^{3/2}}
\]  \hspace{1cm} (3)
where \( \nu \) is the Poisson ratio, \( F \) is the adhesion force, \( \delta \) is the indentation, \( E \) is the Young's modulus, and \( R \) is the radius of the curvature of the AFM tip, respectively.

A Poisson ratio of 0.5 is appropriate for lipid bilayers, cells, and vesicles (Laney et al. 1997; Liang et al. 2004; Radmacher 2002). The sample indentation, \( \delta \), is defined as the difference between the piezo translation and the cantilever deflection since there is an indentation in the sample caused by its softness. The indentation of only 10% of the total sample thickness was considered for the calculation to limit the influence of the underlying substrate.

Figure 7 shows that the keratocytes cultured on modified membrane has a higher adhesion force than that cultured on modified membrane. The adhesion force of keratocytes cultured on modified membrane is \((2328 \pm 523)\mu\text{N}\), whereas the adhesion force on the unmodified membrane is only \((525 \pm 117)\mu\text{N}\). The adhesion force \( (F) \) is relevant to the attractive force of molecules, and \( F \) rises as the increment of attractive force. Since the size of grain is bigger, the adhesion force of the cell cultured on modified membrane is also higher.

Figure 8 shows that the cell cultured on unmodified membrane is significantly stiffer. On average, the stiffness of cell cultured on unmodified membrane \((0.19 \pm 0.016 \text{ mN/m})\) is 2 times than that of the cell cultured on modified membrane \((0.08 \pm 0.022 \text{ mN/m})\).

Figure 9 shows that the Young’s modulus of the cell cultured on modified membrane is higher than that of the cell cultured on unmodified membrane. This indicates that the cell cultured on modified membrane is not easily deformed. It is known that the Young’s modulus is closely associated with the cytoskeleton. The decrease of Young’s modulus may be owing to the damage of cytoskeleton.

Conclusion

To evaluate the prospects of Col/bFGF-p (HEMA-MMA) for artificial cornea, the cytotoxicity of Col/bFGF-p (HEMA-MMA) was investigated in this article. The adhesion assay is carried out first. The results show that the adhesion rate of modified membrane is nearly 90%, whereas the modified only down to 60%. This indicates that the Col/bFGF-p (HEMA-MMA) had better cellular interaction and is suitable for the attachment and growth of keratocytes. Then, AFM is used to comprehensively analyze the keratocytes cultured on different substrate.

From the morphology image of AFM, we can easily find that the two cells are obviously different. The cell cultured on modified membrane represents a fully spread out, spindle-shape morphology, whereas the cell cultured on unmodified membrane exhibits a partially spread, atrophy appearance.
force–distance curves reveal that the adhesion force (2328 ± 523 pN) and Young’s modulus (0.51 ± 0.125 kPa) of the cell cultured on modified membrane are much higher, and the stiffness (0.08 ± 0.022 mN/m) is lower than those of the cell cultured on unmodified membrane. It is known that the morphology and biomechanical properties have a close relationship with the cell state. The results of AFM imply that the cell cultured on the unmodified material is in better state. The cell adhesion assay also shows the cell–material interaction is much improved. That is, the Col/bFGF-p (HEMA-MMA) could support the keratocytes to adhesion, and the attached cell also in better health state. The Col/bFGF-p (HEMA-MMA) may hold great promise to be an appropriate material for artificial cornea.

References


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