Preparation of gelatin scaffold and fibroblast cell culture

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Abstract A gelatin gel sheet was UV-irradiated to raise the gel melting temperature T_m . Three second of T_m increased proportionally to irradiation time t_{UV} . The sheet of which T_m was larger than the culture temperature 37°C was used as a cell culture scaffold. Human fibroblast cells WI-38 adhered to the sheet and dug deeply into it regardless of t_{UV} , while cancer cells WI-38-VA-13 formed spheroids and did not adhere to the sheet individually at high UV dose. The cell selectivity of the scaffold could be used for biological and medical studies.

Keywords fibroblast, cell culture, gelatin gel scaffold, UV-irradiation

Introduction

The melting point of gelatin gels can be raised by cross-linking gelatin molecules in aqueous solutions with UV irradiation [1]. The modified gels can be used for cell culture scaffold, since they are stable at physiological temperature for cell culture [2]. We reported that human fibroblast cells WI-38 can be cultured on a UV-irradiated gelatin microcapsule scaffold. Interestingly, in contrast to the normal cells, cancer cells WI-38-VA-13 could not be grown on the microcapsules [3, 4]. This characteristic property could be used for selective cell culture both *in vivo* and *in vitro*. A sheet having the same characteristics as the microcapsules would be easier to handle for practical purposes. In this study, we have pre-

pared gelatin gel sheets by UV-irradiation, and observed microscopic difference in adhesion of normal and cancer fibroblast cells on the sheet.

2. Materials and Methods

2.1 Preparation of gelatin gel sheet

Porcine gelatin (APH-250, Nitta Gelatin Inc.) was dissolved in Milli-Q water at 40°C at 3 wt% to make a gelatin solution. 0.5 mL of the solution was dripped in a PMMA pipe (24 mmφ × 10 mm) on a Teflon sheet on ice to make the solution gelled. The physical gel obtained was kept in a refrigerator at 10% humidity at 5°C for 2 days. Both sides of the gel sheet was irradiated with UV ray at 3400 μW/cm² of 254 nm (CSL-100C, Cosmo Bio Co. Ltd) for $t_{UV} = 0 - 400$ min. The distance between the light source and the sheet was 5 cm. The resultant chemical gel was hydrated in Milli-Q water in a refrigerator for one day and used for measurements.

2.2 Measurement of melting point

Each gel sheet prepared with UV irradiation for various period t_{UV} was put in test tubes, sealed and set in a water bath at 26°C. The temperature of the bath was then raised at 0.5°C/min. The melting point of the gel sheets was determined as the temperature when fluidity was observed by eyes.

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2.3 Cell culture

Human fibroblast cells (WI-38) and cancer cells (WI-38-VA-13) were purchased from JCRB Cell Bank. The cell culture medium was Minimum Essential Medium Eagle containing 10 v/v% fetal bovine serum, 50 units/ml of penicillin, 50 mg/ml of streptomycin, an appropriate amount of vitamin and amino acid. Cell culture was performed using the gelatin sheets prepared by UV irradiation for t_{UV} in the range 30–400 min. The sheets were sterilized in 70v/v% ethanol, and washed three times in phosphate buffer at pH = 7.4. For fibroblast cell culture, 1 mL of the medium was poured in 6 well microtiter plates for suspension cells. Then the sheet was put on each well. 1 mL of cells whose number concentration was 5×10^4 cells /mL was added. The plate was put on an analog shaker for suspension culture. The cells were cultured under 5% CO₂ atmosphere at 37°C for 6 days. The shape of the cells and cell aggregates after the culture was observed under an inverted microscope.

2.4 Electron microscopy

The sheets prepared with $t_{UV} = 30$ min and 400 min was fixed by 0.25% glutaraldehyde in 0.1 M phosphate buffered solution (PBS, pH7.4) for one day. The sheets were washed with 0.1 M PBS and dehydrated with graded ethanol solutions successively. The sheets were further washed with t-butylalcohol and freeze-dried. Finally Au-Pd (Gold-palladium alloy) was sputtered on them by an ion sputter device (E101, Hitachi). The surface structure of the sheets was observed by a scanning electron microscopy (S-2250N, Hitachi). For observation of cancer cells and the sheet near the interface by transmission electron microscope (TEM), the sheets were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M PBS at 4°C overnight. After this fixation the sheets were postfixed with 2% osmium tetroxide in 0.1 M PBS at 4°C for 2 h, and were dehydrated in graded ethanol solutions (50%, 70%, 90%, and 100%), successively. After dehydration the sheets were infiltrated with propylene oxide (PO) and were put into a 70:30 mixture of PO and resin (Quetol-812: Nisshin EM Co.), then PO was volatilized overnight. The sheets were transferred to a fresh 100% resin, and were polymerized at 60°C for 48 h. The polymerized resin were ultra-thin sectioned at 80 nm with a diamond knife using an ultramicrotome (Ultracut UCT, Leica) and the sections were mounted on copper grids. They were stained with 2% uranyl acetate at room temperature for 15 min. The grid was observed by a TEM (JEM-1400Plus, JEOL Ltd.) at an acceleration voltage of 80 kV. Digital images were taken with a CCD camera (VELETA, Olympus).

3. Results and Discussion

Figure 1 shows the melting temperature as a function of UV irradiation time t_{UV} for each side. The 3/2 power of melting temperature T_m is linear to t_{UV} , i.e.,

$$T_m \propto (1 + \beta t_{UV})^{2/3} \quad (1)$$

where β is a constant which depends only on gelatin weight fraction. This behavior is the same as that of the gelatin gel wall membrane of microcapsules explained by the following theoretical argument [5]. UV irradiation for gelatin induces intermolecular covalent bondings, which results in molecular weight increase. Equation (1) is generally obtained by assuming that (a) the rate of a binding after the UV irradiation is proportional to the number density of the un-reacted functional groups, and (b) the potential energy resulted from a deviation r of a gelatin molecule from its equilibrium position is given by [5]

$$U(r) = \frac{1}{2} k |r|^2 \quad (2)$$

Fig. 1 Melting temperature of gelatin sheet as a function of UV irradiation time. Dotted line denotes 37°C.

Fig. 2 Scanning electron micrograph of gelatin sheet prepared by UV irradiation for 30 min.

Fig. 3 Optical micrographs of normal cells (WI-38) (A) and cancer cells (WI-38-VA-13) (B) prepared with $t_{UV} = 30$ min (a), 40 min (b) and 400 min (c).

Fig. 4 Transmission electron micrographs near the adhesion surface for WI-38. The magnification is 1760 (a), 4780 (b) and 19000 (c). The cells dig into the gel sheet as shown by red arrows.

Fig. 5 Transmission electron micrographs near the adhesion surface (a) and (b) and in the cell aggregates (c) for WI-38-VA-13. The magnification is 598 for (a) and 19000 for (b) and (c).

Here, k is the elastic coefficient of the binding, and is proportional to $P^{2/3}$ in which P is the degree of polymerization of the gelatin molecule. From (a) for the irradiation time t_{UV} , we have

$$P = 1 + \beta t_{UV} \quad (3)$$

Equation (1) is obtained according to Lindeman's law [6] based on (b). The experimental results show more

than 10 min-UV-irradiation is necessary to raise the melting temperature to the culture temperature of 37°C. Figure 2 shows a scanning electron micrograph of the sheet prepared with UV irradiation at $t_{UV} = 30$ min. Slight wavy surface roughness in the order of microns is observed, which could be attributed to an inhomogeneous shrinking and swelling in the process of soaking in ethanol and culture media. No clear difference was observed in the surface structure of the sheets prepared

with different t_{UV} (not shown).

Figure 3 shows the microscopic images for normal fibroblast cells WI-38 (A) and cancer cells WI-38-VA-13 (B) on the sheet prepared by UV irradiation for t_{UV} being 30 min (a), 40 min (b) and 400 min (c). The normal cells are observed to be spreading out homogeneously in plane on the sheet irrespective of t_{UV} . On the other hand, most cancer cells are spreading out in plane on the sheet with $t_{UV} < 30$ min, and they form spheroid aggregates and adhere to the gel sheet with a small contact area on the sheet with $t_{UV} > 40$ min. More detailed information near the interface between the cell or cell aggregates and the sheet prepared with $t_{UV} = 100$ min is shown as a transmission electron micrographs for normal cells WI-38 in Fig. 4 and cancer cells WI-38-VA-13 in Fig. 5. Figure 4(a) and the enlarged one 4(b) shows that WI-38 cells capture the gel sheet and dig deeply into it. Actin fibers are clearly observed between the cell and the sheet in Figure 4(c). On the other hand, large spheroid aggregates of WI-38-VA-13 cells contact with the gel sheet with a small contact area as shown in Fig. 5(a), but do not dig into the sheet. Figure 5(b) shows actin fibers are formed in the contact cells (Fig. 5(b)). Desmosomes are clearly formed inside the spheroid aggregates, as shown in Fig. 5(c). These experimental results show that cancer cells do not adhere individually to the gelatin sheet modified by UV irradiation with $t_{UV} > 40$ min but tend to form aggregates. WI-38-VA-13 cells synthesize only 1/9 amount of fibronectin compared with WI-38 cells [7]. They secrete a limited amount of fibronectin, but aggregate with each other to form a spheroid to grow. The reason of the difference of observed growth behaviors in WI-38 and WI-38-VA-13 is speculated as follows; WI-38-VA-13 cells adhere to scaffolds having many adhesion sites, while they form a spheroid or adhere with a small contact area on scaffolds having a small number of adhesion sites such as UV-irradiated gelatin scaffold in which many adhesion sites are broken by UV irradiation. While WI-38 cells synthesize and secrete much more amount of fibronectin that enable the cells to adhere to

even UV-irradiated gelatin scaffold. It is interesting to examine the cell selectivity characteristics of UV-irradiated gelatin gel sheet for cells of different kinds.

In conclusion, the relationship between UV irradiation time for preparing gelatin gel sheet and gel melting temperature was determined and explained by Lindeman's law. The UV-irradiated gelatin gel scaffold was applied to culture normal fibroblast cells WI-38 and cancer cells WI-38-VA-13. Optical and electron micrographs demonstrated that the cancer cells formed spheroid aggregates on the sheet prepared with large irradiation time but most cancer cells did not adhere to the sheet, in contrast to homogeneous adhesion of normal cells to the scaffold.

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References

1. For example, Otoni CG, Avena-Bustillos RJA, Chiou BS, Bilbao-Sainz C, Bechtel PJ, McHugh TH. Ultraviolet-B radiation induced cross-linking improves physical properties of cold- and warm-water fish gelatin gels and films. *J Food Sci.* 2012; 77: E215–23.
2. Kang HW, Tabata Y, Ikada Y. Fabrication of porous gelatin scaffolds for tissue engineering. *Biomaterials.* 1999; 20: 1339–44.
3. Dobashi T, Koike M, Kobayashi K, Maki Y, Yamamoto T, Tanaka S. An application of microcapsules having enzyme-degradable gel membrane to cell culture. *Progr Colloid Polym Sci.* 2009; 136: 149–54.
4. Koike M, Kobayashi K, Tanaka S, Harano A, Yamamoto T, Dobashi T. Development of cell culture scaffold composed of microcapsule: determination of preparation condition. *Trans MRS-J.* 2006; 31: 823–5.
5. Yamamoto T, Koike M, Dobashi T. Melting and swelling behaviors of UV-irradiated gelatin gel microcapsules. *Langmuir.* 2007; 23: 8531–7.
6. Lindeman FA. The calculation of molecular vibration frequencies. *Physik Z.* 1910; 11: 609–12.
7. Lee BH, Park RW, Sohn KY, Kim IS. Antagonistic regulation of protein kinase C-induced stimulation of fibronectin synthesis by cyclic AMP in human lung fibroblasts. *Biochem Mol Biol Int.* 1997; 43: 383–90.