Micropattern-immobilization of heparin to regulate cell growth with fibroblast growth factor

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Abstract

Heparin was immobilized on a polystyrene plate in a specific pattern by photolithography. Heparin was coupled with azidoaniline. The derivatized heparin was cast on the polystyrene plate from aqueous solution. After drying, the plate was photo-irradiated in the presence of a photomask. The micropatterning was confirmed by staining with a dye, ethidium bromide. Since heparin has negative charges, the cationic dye was adsorbed on the regions where heparin was immobilized. In the presence fibroblast growth factor (FGF), the growth of mouse fibroblast STO cells was enhanced only on the heparin-immobilized regions. This result indicated that micropattern-immobilized heparin activated FGF for cell growth activity.

Abbreviations: FGF, fibroblast growth factor

Introduction

One of the most widely employed approaches in the synthesis of blood compatible materials is heparinization, in which synthetic polymers are coated or immobilized with heparins (Ito, 1987). Heparins are a linear anionic polysaccharide chain; they are typically heterogeneously sulphated on alternating L-iduronic acid and D-glucosamino sugars, and are nearly ubiquitous in animal tissues as heparan sulphate proteoglycans on cell surfaces and in the extracellular matrix (Casu, 1995). Recently it was reported that the activation of fibroblast growth factor (FGF), which form a large family of structurally related, multifunctional proteins that regulate various biological responses, require heparin-like molecules (Ornitz et al., 1992; Faham et al., 1996; Digrafi et al., 1998).

We have micropattern-immobilized various types of bioactive macromolecules to regulate cell functions (Ito et al., 1996, 1997a, b, c, 1998a; Chen et al., 1997). Immobilization of growth factor proteins induced cell

growth or differentiation according to 'artificial juxtacrine stimulation' (Ito, 1998; Ito et al., 1998b). The micropattern-immobilization was useful to visualize cellular signal transduction and to confirm the completeness of immobilization of bioactive macromolecules. In addition to proteins, sulphated hyaluronic acid was immobilized and it was found that the immobilized polysaccharide reduced platelet adhesion by the micropatterning technique (Chen et al., 1998). In the present study, heparin was micropattern-immobilized on a polystyrene plate to visualize the interaction with cell. This is the first report to clearly show the effect of immobilized heparin on cell behavior.

Materials and methods

Materials

Heparin sodium salt (081-00136), 4-azidoaniline hydrochloride, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (water-soluble carbodiim-

Figure 1. Micropattern-immobilization of azidophenylamino-derivatized heparin (AzHep).

ide, WSC), and brilliant green were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Basic FGF (bFGF) was purchased from Toyobo Co., Ltd (Osaka, Japan). Photo-lithographic mask was purchased from Nippon Filcon Co., Ltd. (Osaka, Japan). Polystyrene plate was obtained by cutting the cell culture dish purchased from Akita Sumitomo Bake Co. (Akita, Japan).

Synthesis of photo-reactive-heparin

Heparin (18.2 mg), 4-azidoaniline (7.75 mg), and WSC (10 mg) were dissolved in deionized water (20 ml) and the pH of the solution was adjusted to 7.0. The solution was stirred at 4 °C for 24 h. Then, the solution was ultrafiltrated (Millipore MoleCut II, filtration off below M.W. 3,000) and the residue was repeatedly washed with distilled water until the absence of 4-azidoaniline in the filtrate was confirmed by ultraviolet absorption at 257 nm. The purified solution was then lyophilized. The photo-reactive heparin conjugate is referred to as AzHep. The content of azidophenyl groups in the heparin was measured by ultraviolet absorption.

Figure 2. UV spectra of (a) 4-azidoaniline (3 µg/ml), (b) AzHep (100 µg/ml) and (c) original heparin (100 µg/ml).

Photo-immobilization

The micropattern-immobilization was carried out according to the method reported by Matsuda and Sugawara (1995a, b). The process is shown in Figure 1. An aqueous solution of AzHep (1 mg/ml, 0.1 ml) was dropped on a polystyrene plate air-dried at room temperature. Subsequently the plate was irradiated with an ultraviolet lamp (Koala, 100 W) at a distance of 5 cm for 30 s in the presence or absence of a photomask. The plate was washed with distilled water until the absence of AzHep in the wash was confirmed by ultraviolet spectroscopy. The plate was immersed in an aqueous solution of ethidium bromide (1 mg/ml) for 5 min to be stained. After washing with distilled water, the stained plate was observed using a laser microscope (Olympus Co., Tokyo, Japan).

Cell culture

Mouse fibroblast STO cells (Ito et al., 1991) were subcultured in Dulbecco's modified Eagle minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum in air containing 5% CO₂ at 37 °C. After the cells were cultures in the absence of serum for 2 days, they were harvested by incubation at 37 °C for 10 min in PBS containing 0.15% (w/v) trypsin and 0.2% (w/v) ethylenediamine tetraacetic acid and by

Figure 3. Micrographs of (a) photo-mask and (b) AzHep-immobilized film. AzHep was stained by ethidium bromide.

subsequent pipetting. After being washed twice with MEM medium, the cells were suspended in MEM medium (1×10^5 cells/ml) containing $1.0 \mu\text{g/ml}$ bFGF. The cell suspension was added on the polystyrene

plates and cultured in air containing 5% CO_2 at 37°C . The cultured cells were observed by phase-contrast microscopy.

72 h $\downarrow$ +bFGF

Mouse Fibroblast STO Cells

Figure 4. Cells grown on the polystyrene film micropattern-immobilized with AzHep.

Figure 5. Schematic drawing of enhancement of cell growth on a heparin-immobilized region in the presence of FGF. Immobilized heparin was considered to induce dimerization of FGF.

Results and discussion

Ultraviolet spectra of Hep and AzHep are shown in Figure 2. In the spectrum of AzHep, an absorption at 269 nm, which is assignable to azidophenyl group was observed. The absorption somewhat red-shifted from the corresponding absorption of 4-azidoaniline. This shift could be due to electron delocalization of azidophenyl group by the amide bond formation. Assuming that the molecular adsorption coefficient of azidophenyl group at 268 nm was the same as that of 4-azidoaniline at 263 nm, the content of azidophenyl groups in the heparin was about 0.05 mole/mole saccharide unit. It is known that two carboxyl groups exist in a tetrasaccharide unit of heparin. One of ten carboxyl groups was coupled with azidophenyl group.

Micropattern-immobilization of AzHep

AzHep was photo-immobilized in the presence of a photo-mask. Upon UV irradiation, the azidophenyl group was easily photolyzed to generate highly reactive nitrene, which spontaneously formed covalent bonds with neighboring hydrocarbon in the polystyrene film surface. After thoroughly washed with distilled water, the immobilized polymer was exactly in the pattern of the photo-mask as shown in Figure 3. Since the polymer was anionic, the cationic dye, brilliant green, selectively stained the areas of AzHep immobilization.

Cell growth

Cells before and after 48 h culture on the micropattern-immobilized AzHep are shown in Figure 4. The cells adhered on the plate independently of the heparin-immobilized regions. However, the growth of cells

were observed only on the regions immobilized with heparin. This result demonstrates that the cell growth was stimulated by FGF interacting with heparin as shown in Figure 5.

The micropattern-immobilization enabled visual evaluation of biological activities of hybrid material under the same conditions as for non-treated material. In the present investigation, the importance of heparin for FGF activation was visualized by the micropattern-immobilization of heparin.

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