Patterned artificial juxtacrine stimulation of cells by covalently immobilized insulin

Yoshihiro Ito[a,b,*], Satoshi Kondo[b], Guoping Chen[b], Yukio Imanishi[b]

[a]*PRESTO, JST, Keihanna Plaza, 1-7 Seika-cho, Kyoto 619-02, Japan*
[b]*Department of Material Chemistry, Graduate School of Engineering, Kyoto University, Kyoto 606-01, Japan*

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Abstract Insulin was immobilized on prescribed areas of poly(ethylene terephthalate) film by photolithography. Chinese hamster ovary cells overexpressing human insulin receptors were cultured on the pattern-immobilized film. Pattern-immobilized insulin did not enhance cell adhesion but transduced a signal to the cells through phosphorylation of tyrosine residues of signal proteins. In addition, only cells on immobilized insulin grew in medium containing no serum. The enhancement of cell growth was considered to be a consequence of signal transduction. This microprocessing technique is useful for investigating the effect of immobilized biosignal molecules and for constructing new tissue engineering materials.

Key words: Juxtacrine stimulation; Patterned immobilization; Tissue engineering; Growth factor; Insulin

1. Introduction

Growth factors are generally secreted as diffusible proteins and transduce proliferation and differentiation signals, while cell adhesion molecules link to extracellular matrices and assemble animal cells into tissues through their adhesive properties. However, these two communication systems substantially overlap [1–3]. For example, integrins are receptors for cell adhesion molecules, both providing a physical link to the cytoskeleton and transducing signals such as migration, morphogenesis, growth, differentiation and apoptosis from the extracellular matrix [4–6]. In addition, several growth factors have been reported to regulate cell functions in the transmembrane form by 'juxtacrine stimulation' [7–10]. The mechanism of stimulation was deduced from studies of intercellular regulation by paraformaldehyde-fixed cells that express the growth factors or cytokines.

On the other hand, we showed that immobilized insulin enhanced growth of anchorage-dependent cells to a greater extent than soluble protein [11]. The immobilized insulin activated the insulin receptor and downstream signaling proteins, and this activation persisted for longer periods than that with soluble insulin, probably explaining the greater mitogenic effect of the immobilized insulin [12]. In this study, signal transduction from the immobilized insulin was clearly visualized and the possibility of regulating cell functions through 'artificial juxtacrine stimulation' was examined using a micro-pattern-immobilized insulin. The patterned immobilization method provides a new valuable tool to detect biosignal molecule-sensitive cells, to investigate cooperative or competitive effects between immobilized and non-immobilized biosignal molecules, or to regulate tissue formation on matrices.

2. Materials and methods

2.1. Preparation of pattern-immobilized insulin

Fig. 1 shows the preparative scheme of pattern-immobilized insulin. Poly(acrylic acid) carrying phenylazido groups (AzPhPAA) was synthesized and AzPhPAA was immobilized on a prescribed region by

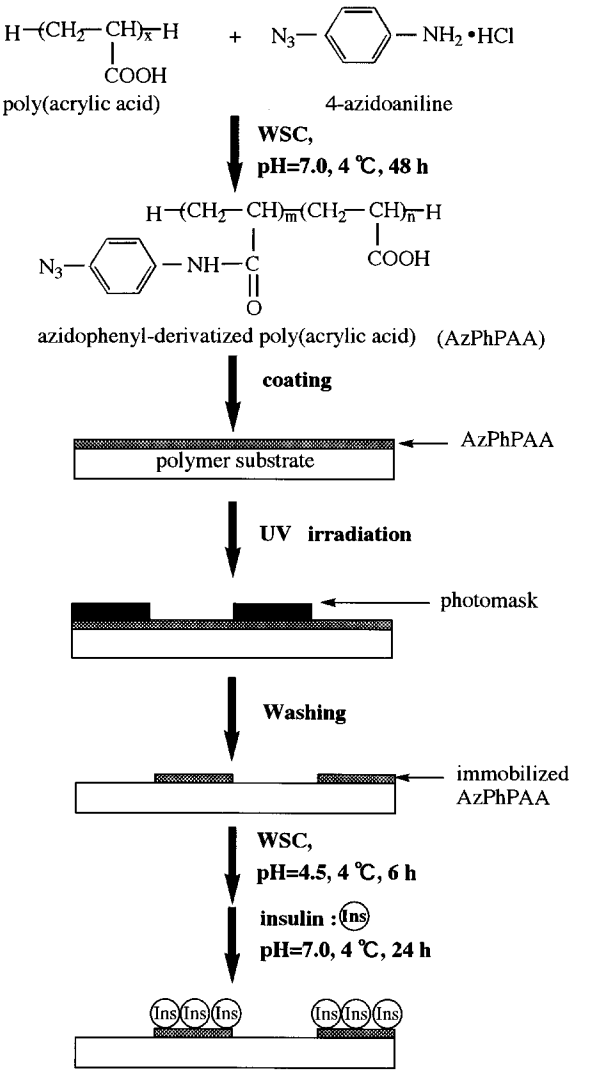

Fig. 1. Preparative scheme of patterned-immobilized insulin. Photoreactive polymer was synthesized and then pattern-immobilized on a polymer film by photolithography. Insulin was immobilized on the pattern-immobilized polymer carrying carboxylic acid in the side chains.

*Corresponding author. Fax: (81) (75) 753 4911.
E-mail: h51535@sakura.kudpc.kyoto-u.ac.jp

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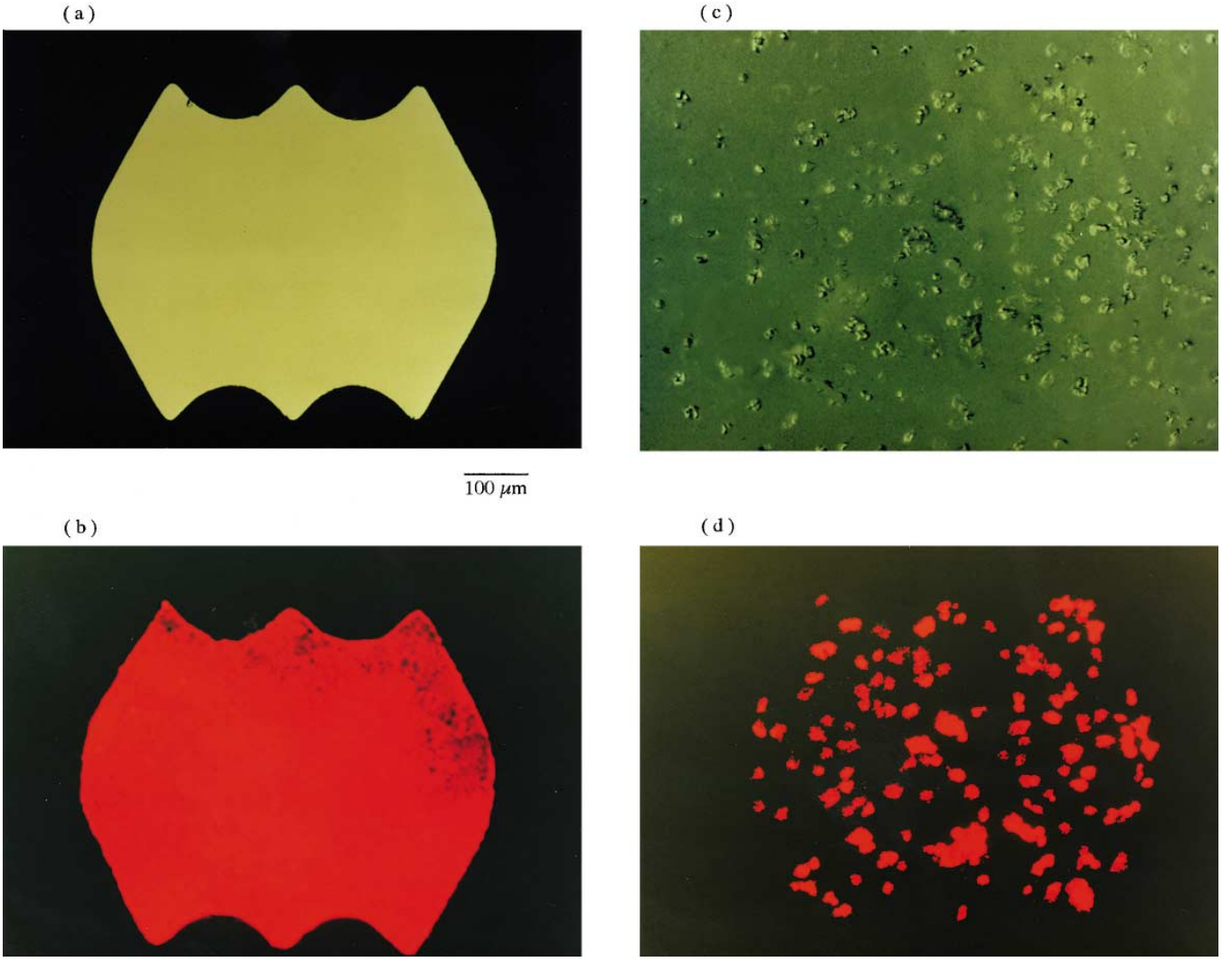

Fig. 2. Micrograph of photomask (a), fluorescence micrograph of immobilized insulin stained by anti-insulin antibody (b), optical micrograph of adhered CHO-T cells cultured for 8 h (c), and fluorescence micrograph of the CHO-T cells stained by anti-phosphotyrosine antibody (d). (c,d) The same spot observed by transmission and fluorescence microscopy, respectively.

photolithography. Insulin was immobilized on the AzPhPAA-immobilized area using water-soluble carbodiimide as a coupling agent.

Poly(acrylic acid) carrying azidophenyl groups was synthesized as reported by Sugawara and Matsuda [13]. Poly(acrylic acid) (MW = 450 000, 1.0 mmol of monomer unit), 4-azidoaniline (0.2 mmol), and 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (water-soluble carbodiimide, WSC, 6.0 mmol) were dissolved in deionized water (110 ml) and stirred for 48 h at 4°C. After the reaction mixture had been concentrated under reduced pressure, dialysis was conducted using a seamless cellulose tube (cut off molecular weight of 12 000). The dialyzed polymer was freeze-dried in vacuo to obtain a white solid (yield 47.2%). The degree of substitution (15%) of the phenylazido group was determined by ^{1}H-NMR from the peak intensities of the phenylazido protons at 7 ppm and those of the methylene protons of the AzPhPAA main chain at 1.3 ppm.

A water/methanol (2:3 v/v) mixed solution containing AzPhPAA (0.5 wt%) was cast on a poly(ethylene terephthalate) film (diameter, 13.5 mm) and air-dried. The film was covered with a patterned photomask and was UV-irradiated for 10 s using a UV lamp (Koala, 100 W) at a distance of 5 cm. The film was thoroughly washed with phosphate-buffered saline (PBS). The film was immersed in a 2-(*N*-morpholino)ethanesulfonate-buffered solution (pH 4.5, 0.1 M, 10 ml) containing WSC (1 mg/ml) for 6 h at 4°C. After being washed, the film was incubated with insulin solution (1 mg/ml, 10 ml) for 24 h at 4°C. The immobilized insulin film was washed with PBS until no release of insulin was detected by ultraviolet spectroscopy at 280 nm.

The pattern-immobilized film was immersed in PBS containing 0.02% NaN_3 and 3% bovine serum albumin (BSA) for 12 h at 4°C. The film was then incubated in PBS containing anti-insulin mouse IgG antibody (Kyowa Medics, 5 μg/ml) and 0.02% NaN_3 for 3 h at 4°C. After being washed with PBS containing 0.02% NaN_3, the film was incubated in PBS containing rhodamine-labeled anti-mouse IgG antibody (Protos, 5 μg/ml) and 0.02% NaN_3 for 2 h at 4°C. The stained film was washed with PBS and observed under a fluorescence microscope (Olympus).

2.2. Cell culture

Chinese hamster ovary cells overexpressing insulin receptors (CHO-T, 10^6 receptors per cell) [12] were subcultured in Ham's F-12 medium containing 10% (v/v) fetal bovine serum under 5% CO_2 in air at 37°C. After culture in the absence of serum for 2 days, cells were harvested by incubation at 37°C for 10 min with PBS containing 0.02% (w/v) EDTA, washed with Ham's F-12 medium twice, and suspended in Ham's F-12 medium (1×10^5 cells/ml). The suspension was placed on a sample film and incubated under 5% CO_2 in air at 37°C.

The cells on film were treated for observation by fluorescence microscopy as follows. After 8 h culture, the film with adherent cells was incubated in 4% paraformaldehyde for 1 h at 4°C. The film was washed three times with PBS and incubated in PBS containing 0.25% Triton X-100 and 1 mM Na_3VO_4 for 30 min at 4°C. After

being washed three times with PBS containing 1 mM Na_3VO_4, the film was incubated in an aqueous solution containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.01% Tween 20, 0.02% NaN_3, 1 mM Na_3VO_4 and 3% BSA for 12 h at 4°C. This was followed by incubation in PBS containing anti-phosphotyrosine mouse IgG antibody (Santa Cruz, 1 μg/ml) for 3 h at 4°C and washing three times with an aqueous solution (washing solution) containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.01% Tween 20, 0.02% NaN_3, 1 mM Na_3VO_4. Subsequently, the film was incubated in PBS containing rhodamine-labeled anti-mouse IgG antibody (2 μg/ml) for 2 h at 4°C. After being washed three times with the washing solution, three times with PBS, and rinsed briefly with distilled water, the stained film was mounted in Vectashield mounting medium and observed by microscopy.

3. Results and discussion

The patterned immobilization of insulin using a photomask (Fig. 2a) was observed by anti-insulin antibody staining as shown in Fig. 2b. CHO-T cells were cultured on the film on which insulin was pattern-immobilized in the absence of serum. The cells adhered on the film independently of the immobilized insulin area after 8 h of culture (Fig. 2c). However, only the cells on the immobilized insulin area were stained by the anti-phosphotyrosine antibody (Fig. 2d). These findings indicated that the immobilized insulin did not enhance cell adhesion but transduced a signal to the cells through phosphorylation of tyrosine residues of signal proteins. Previously, we reported that immobilized insulin activated insulin receptors, insulin receptor substrate-1 and phosphatidylinositol-3-kinase for a longer period than free insulin [12]. Therefore, even after 8 h of culture, activated cells were observed on the insulin immobilized region. This phenomenon was considered to be due to the inhibition of receptor internalization (inhibition of down-regulation). In addition, it was considered that the potential interactions of regulators such as tyrosine phosphatases and serine/threonine-dependent protein kinases were inhibited by the prevention of lateral diffusion of activated receptors in the plane of the membrane.

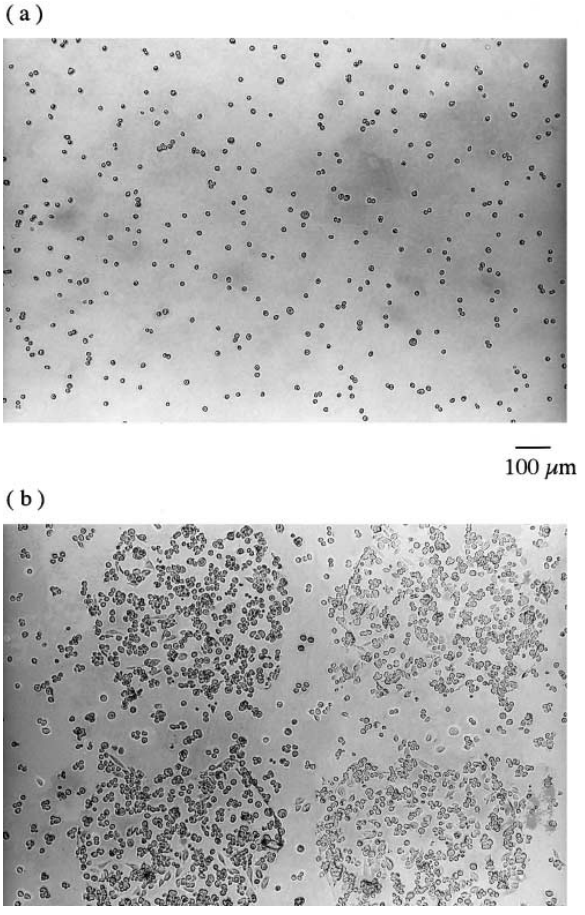

Fig. 3. Phase-contrast micrograph of CHO-T cells grown on the film before (a) and after (b) 3 days. Subcultured CHO-T cells were suspended in Ham's F-12 medium (1×10^5 cells/ml) and cultured on the immobilized insulin film under 5% CO_2 in air at 37°C.

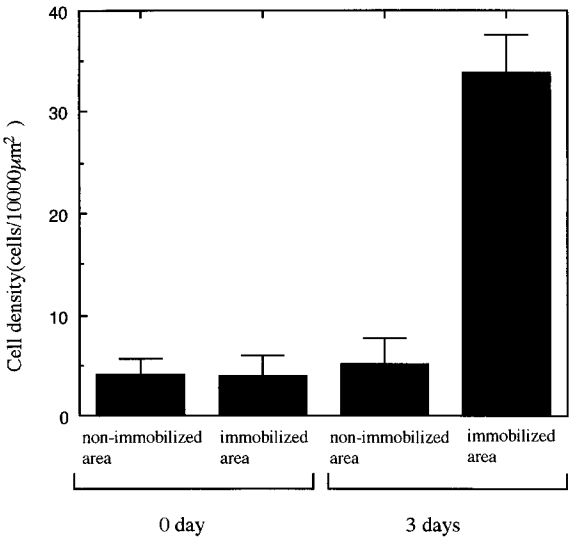

Fig. 4. Cell density in the areas with immobilized and non-immobilized insulin before and after cultivation. The cell density was counted under a phase-contrast microscope. $n=30$. Bars represent standard deviations.

Fig. 3 shows that only the cells on the immobilized insulin grew, although the percentage of spread cells was low because of the absence of serum. The enhancement of cell growth was considered to be the result of signal transduction. Fig. 4 compares the cell density in areas with immobilized insulin and non-immobilized insulin before and after culture. The increase in the number of cells on the immobilized insulin area is not caused by cell migration from the non-immobilized area to the immobilized area.

The interaction of immobilized biosignals with cells was investigated by fixing insulin [14] and nerve growth factor [15] to microporous polysaccharide gels. However, the usefulness was limited by detachment of the biosignal molecules from the gel [16–18]. On the other hand, this pattern-immobilized insulin directly showed the signal transduction from the immobilized insulin by indicating pattern-formation of cells. Recent studies on overlapping of adhesion molecules and growth factors [5], the growth stimulation by non-internalizing epidermal growth factor receptors [19], and various juxtacrine stimulators [7–10], suggest that the signal of immobilized insulin was transduced and cell growth stimulated without internalization. By using the biological activity of immobilized biosignal molecules, it will be possible to design and synthesize new tissue engineering materials. Co-immobilization of different biosignal molecules such as growth factors

and adhesion molecules or immobilization of different density of biosignal molecules is useful to regulate the cell functions.

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