Design and Synthesis of Hybrid Biomaterials for Cell-Culture Engineering

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CONTENTS

LIST OF ABBREVIATIONS

AA	acrylic acid
ABC	avidin-biotinylated horseradish peroxidase complex
AIBN	2,2'-azobisisobutyronitrile
AzPhPAA	azidophenyl-derivatized PAA
AzPhPAAm	azidophenyl-derivatized polyallylamine
AzPhPAAmEGF	azidophenyl-derivatized polyallylamine-EGF conjugate
AzPhPIA	azidophenyl-derivatized PIA
BSA	bovine serum albumin
CHO	Chinese hamster ovary
CHO-ER	CHO cell overexpressing EGFR
CHO-T	CHO cell overexpressing insulin receptors
cpm	count per minute
DAB	diaminobenzidine
DCC	dicyclohexylcarbodiimide
DMF	*N,N*-dimethylformamide
DMEM	Dulbecco's modified Eagle's minimum essential medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGF-Fn-PSt	PSt film coimmobilized with Fn and EGF
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid
ESCA	electron spectroscopy for chemical analysis
FBS	fetal bovine serum
Fn	fibronectin
Fn-PSt	Fn-immobilized PSt film
HPLC	high performance liquid chromatography
^{3}H-thymidine	^{3}H-labeled thymidine
^{125}I-insulin	^{125}I-labeled insulin
^{125}I-EGF	^{125}I-labeled EGF
Ins-PAA	insulin-PAA conjugate
Ins-POE	insulin-POE conjugate
Ins-PSt	insulin-immobilized PSt film

LCST	lower critical solution temperature
MAP	mitogen-activated protein
MBP	myelin basic protein
MES	2-(*N*-morpholino)ethanesulfonic acid
MW	molecular weight
MPA	mercaptopropionic acid
NGF	nerve growth factor
NIPAAm	*N*-isopropylacrylamide
PAA	poly(acrylic acid)
PAA-PSt	PAA-grafted PSt film
PAAm	polyallylamine
PBS	phosphate-buffered saline solution
PBS(-)	PBS without Ca^{2+} and Mg^{2+}
PET	poly(ethylene terephthalate)
PIA	NIPAAm/AA copolymer
PMSF	phenylmethylsulfonyl fluoride
PNIPAAm	poly(*N*-isopropylacrylamide)
PNIPAAm-PSt	PNIPAAm-grafted polystyrene
POE	polyoxyethylene
PSt	polystyrene
RT	room temperature
TBS	Tris-buffered saline solution
TBST	TBS containing Triton X-100
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIP	transferrin, insulin and progesterone
TMPOE	tresylated ω-methoxypolyoxyethylene
Tris	tris(hydroxymethyl) aminomethane
UV	ultraviolet light
WSC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, water-soluble carbodiimide

INTRODUCTION

Biomaterials are important for fabrication of artificial organs and for biochemical processes to produce biological substances by cell-culture engineering. For example, materials have been designed to replace soft and hard tissues and to act as scaffold of cells[1,2]. Approaches for controlling interactions at the interface between living tissues or living cells and materials can be summarized in two principles. One is chemical modification and the other is surface biolization.

The first chemical approach contains surface hydrophilization or surface hydrophobilization of polymer film by chemical[3-5] or plasma treatment[6-8]. Although these methods of chemical modification were not satisfactory in producing biocompatible materials, more precise control of surface properties has become possible after recent progress in polymer modification. The geographic location of cells on the film surface can be regulated by photo-lithographic modification of the surface[9], and the attachment and detachment of cells to/from the film surface can be controlled by the action of thermo-responsive polymer[10,11].

The second biological approach contains immobilization of anticoagulant or hybridization of endothelial cells for blood-compatible materials, and immobilization of cell-adhesion peptide for tissue generation[12,13]. In addition, it has recently been demonstrated that immobilized cell-growth factor enhances cell growth[14]. It is expected that immobilized biosignal proteins might control cell behaviors such as growth, differentiation, secretion, and mobility.

The development of elaborate biomaterials to control cell behaviors has been longed for. The present author has been interested in synthesizing this kind of biomaterials either by biological method or by chemical method. In the present investigation, the present author took aim at synthesizing biological hybrid materials to control cell behaviors such as growth and differentiation, chemical hybrid materials to control cell adhesion, and also chemical/biological hybrid materials to control geographic distribution of cells for tissue generation. The experimental results are summarized in this thesis, which contains three parts and eight chapters.

In Part I of the present thesis, suface biolization of polymer film was carried out by immobilization of cell growth factors, insulin and epidermal growth factor (EGF), and by using the biological hybrid materials, cell growth was accelerated and cell differentiation was promoted (Figure 1).

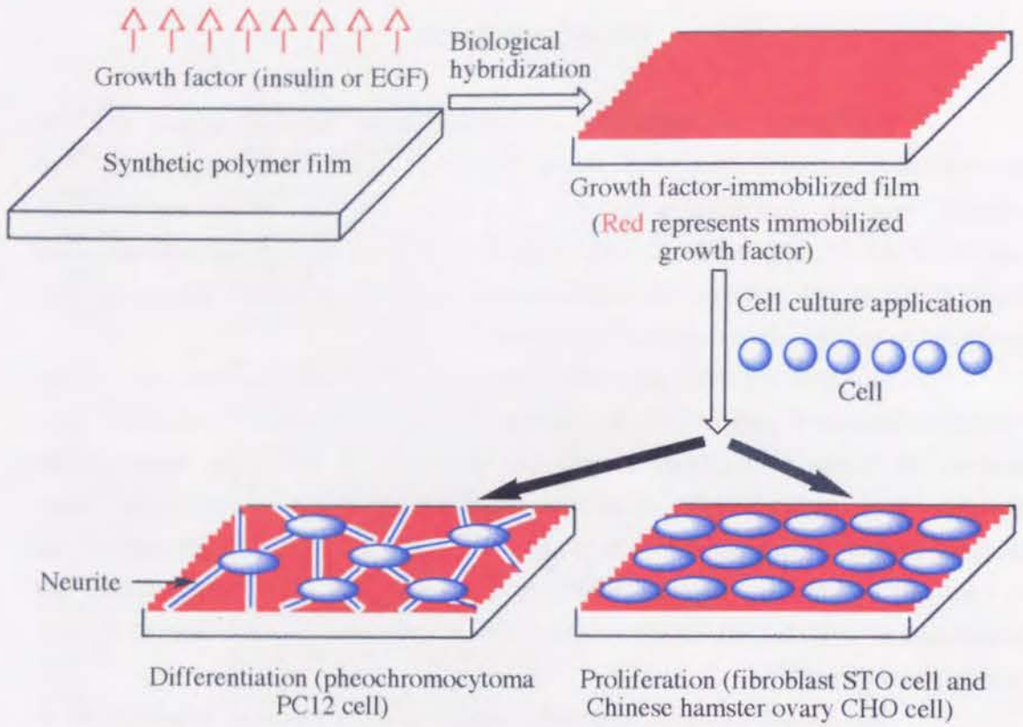

Figure 1 Biolization of synthetic polymer film with biosignal proteins to control cell growth and cell differentiation.

In chapter 1, several insulin derivatives such as immobilized insulin and insulin conjugates with soluble polymers were synthesized, and their mitogenic effect on mouse fibroblast STO cell was compared with that of native insulin. Insulin coupled with polyoxyethylene was a water-soluble monovalent conjugate. Multivalent insulin conjugates were synthesized by coupling insulin with poly(acrylic acid) or poly(acrylic acid)-grafted polystyrene film. The former was soluble in water but the latter was insoluble. The effect of multivalency of insulin conjugates on their mitogenic activity was investigated. In addition, the effect of immobilization of insulin onto insoluble matrix was also studied. The water-soluble multivalent insulin conjugate showed higher mitogenic activity than the native insulin, and the immobilized insulin showed the highest mitogenic activity.

In chapter 2, insulin was photo-immobilized on a polystyrene plate. Since the high mitogenic activity of immobilized insulin was demonstrated in chapter 1, a method for facile preparation of immobilized insulin was devised. Photo-reactive insulin was synthesized by coupling insulin with azidobenzoic acid. Insulin was immobilized on a polystyrene culture plate by coating the insulin derivative on the wells of the plate and a

subsequent photo-irradiation. The effect of photo-immobilization of insulin on the growth of anchorage-dependent cells such as Chinese hamster ovary CHO-K1 cell and mouse fibroblast STO cell was investigated. Photo-immobilized insulin maintained high mitogenic activity for these cells.

In chapter 3, EGF was photo-immobilized on a polystyrene plate by the same method as described in chapter 2. Anchorage-dependent and anchorage-independent cells were cultured on the plate. It has been reported that EGF is a member of membrane-bound growth factors which stimulate cells without diffusion in medium[15]. This type of stimulation is named "juxtacrine stimulation". The photo-immobilized EGF was considered to be an "artificial juxtacrine stimulation". The possibility to use immobilized biosignal molecules to investigate juxtacrine stimulation was investigated.

In chapter 4, the relationship between the stimulation mode of biosignal molecule and the biological activity was investigated. It was reported that the immobilized EGF stimulated a signal protein kinase, mitogen-activated protein (MAP) kinase for longer time than native EGF, resulting in enhancement of growth of Chinese hamster ovary cell[16]. On the other hand, it is known that switching of growth and differentiation of pheochromocytoma PC12 cell depends on the period of activation of MAP kinase[17-21]. Therefore, PC12 cell was cultured on immobilized EGF to investigate the effect of soluble (native) and insoluble (immobilized) EGFs on the differentiation of PC12 cell. Immobilized EGF could switch PC12 cell from growth to differentiation.

In Part II of this thesis, thermal control of cell adhesion and detachment was investigated by using thermo-responsive polymer (Figure 2).

In chapter 5, thermo-responsive polymer, poly(*N*-isopropylacrylamide) (PNIPAAm), was grafted in a prescribed pattern on a polystyrene substratum by photo-lithography. *N*-Isopropylacrylamide was copolymerized with acrylic acid and the copolymer was coupled with azidoaniline. The lower critical solution temperature (LCST) of the copolymer and the derivative was measured. The micro-patterning was carried out by photo-lithography using a photomask. The effect of temperature on the wettability of surface grafted with PNIPAAm was investigated by measuring contact angle. The micro-pattern on the surface at a temperature higher than LCST was observed by phase-contrast microscopy. By culturing mouse fibrobalst STO cell on the micro-patterned plate, the selective detachment of attached cells from regions grafted with PNIPAAm was investigated by lowering temperature.

In chapter 6, insulin was immobilized on PNIPAAm-grafted polystyrene plate to attain enhancement of cell growth as well as easy recovery of cells by lowering temperature. First, the effect of insulin coupling on the LCST of the thermo-responsive

polymer, poly(*N*-isopropylacrylamide-*co*-acrylic acid), was investigated. Subsequently, the thermo-responsive polymer was grafted to glow-discharged polystyrene film and covalently conjugated with insulin. And then, the temperature dependence of surface wettability of the conjugate film was studied by measuring contact angle. Finally, the mitogenic activity of immobilized insulin and the recovery of cells cultured on the film by lowering temperature were examined. In addition, the viability of recovered cells was investigated.

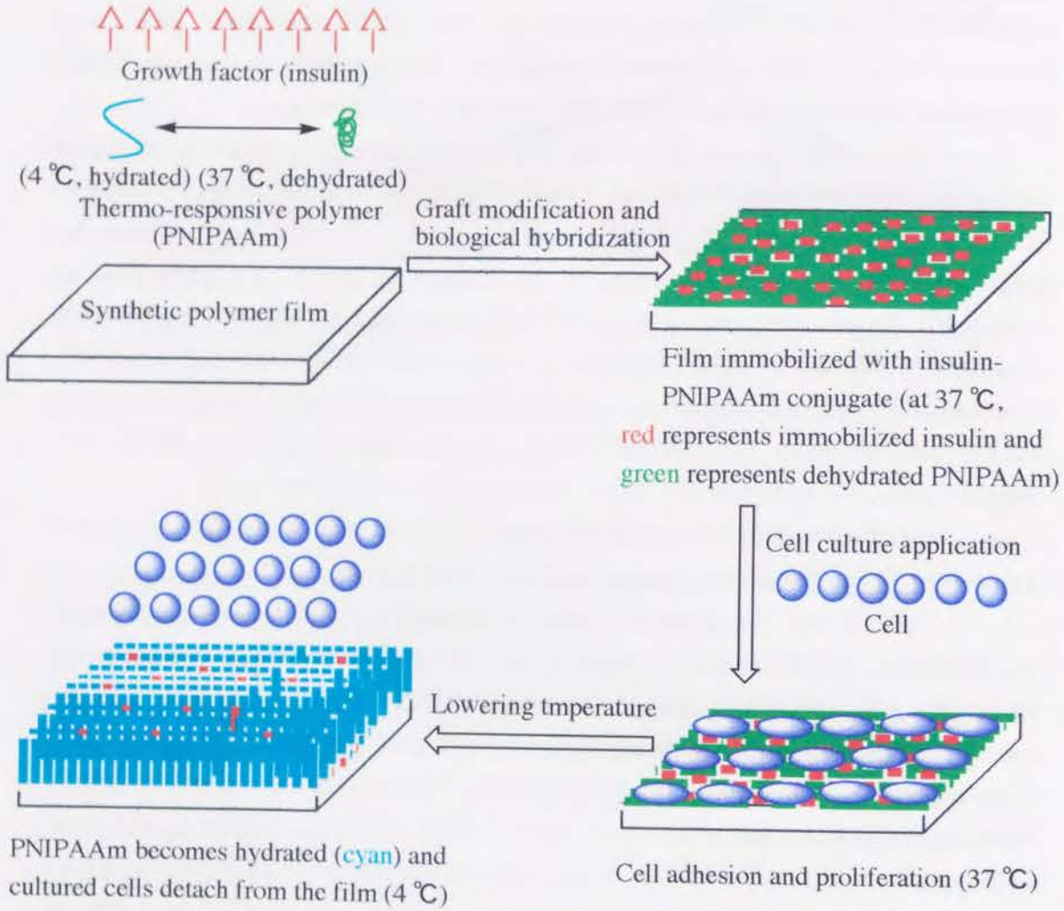

Figure 2 Synthesis of thermo-responsive biomaterials to control cell adhesion and detachment.

In Part III of the thesis, new biomaterials were designed and synthesized for two-dimensional control of cell growth by combination of photo-lithography and biological hybridization (Figure 3).

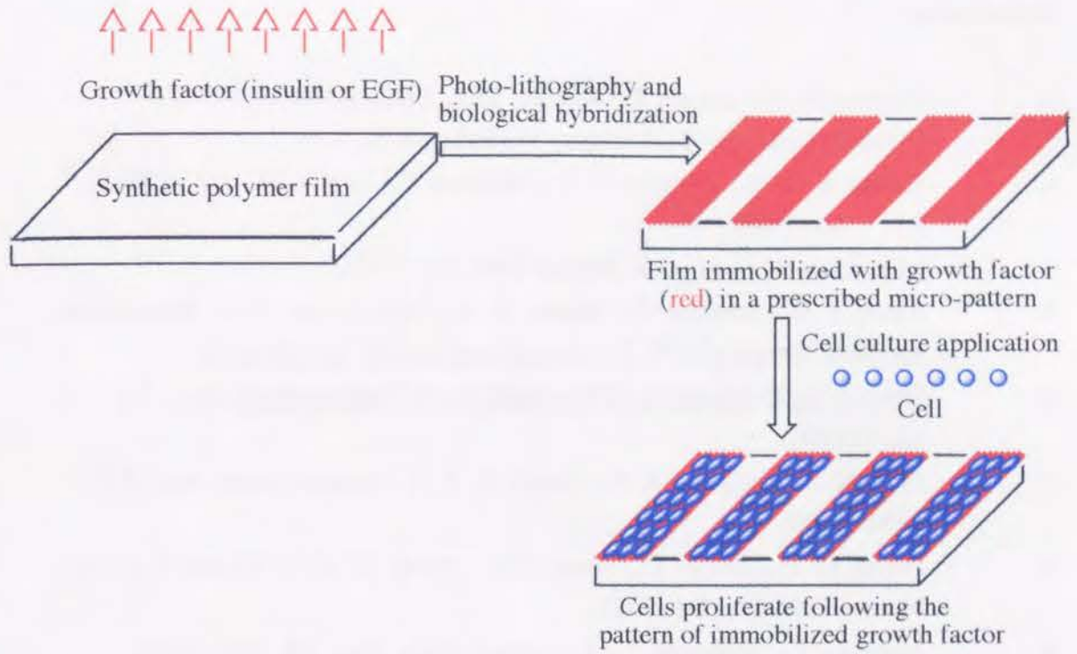

Figure 3 Synthesis of chemical/biological hybrid materials for geographic control of cell growth.

In chapter 7, insulin was immobilized in a prescribed pattern on a poly(ethylene terephthalate) (PET) film for regulation of cell growth through patterned artificial juxtacrine stimulation. Photo-reactive poly(acrylic acid) was synthesized and grafted in a prescribed pattern on a PET film. Insulin was then covalently immobilized only on the poly(acrylic acid)-grafted regions of the film surface. Chinese hamster ovary cells overexpressing insulin receptors, which proliferated on the immobilized insulin regions, were stained by anti-phosphotyrosine antibody. The relationship between the pattern of immobilized insulin and the pattern of grown cells was investigated.

In chapter 8, EGF was immobilized in a prescribed micro-pattern on a polystyrene plate and Chinese hamster ovary cells overexpressing EGF receptors (CHO-ER cells) was cultured on the plate. The phosphorylated tyrosine residues in the cultured CHO-ER cells were examined by immunofluorescence microscopy. The cells adhered on the plates immobilized with EGF in patterns of 2-μm width, which is smaller than the size of cell, or 100-μm width, which is larger than the size of cell, were examined to investigate the signal transuduction triggered by the complex formation of immobilized EGF and EGF receptor.

The details of the experimental results are described in following chapters.

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Part I

Surface Biolization of Polymer Film: Immobilization of Growth Factor

Chapter 1

Mitogenic Activities of Water-Soluble and Water-Insoluble Insulin Conjugates

Introduction

Developments in molecular biology have disclosed the importance of biosignalling polypeptides, including growth factors and cytokines. The covalent binding of biosignal molecules to a soluble or insoluble matrix has a wide range of applications ranging from the elucidation of biosignaling mechanisms and receptor isolation to clinical therapeutics such as drug delivery and tissue regeneration systems[1-4]. The binding of biosignal molecules to their specific receptors may form specific complexes which are assembled and internalized into the cytoplasm with a portion of the receptors returning to the cell surface (down-regulation). However, whether or not all of these processes are necessary has not been clarified. If the internalization of biosignal molecule/receptor complexes is not indispensable for signal transduction, biosignal molecules covalently linked to water-insoluble matrix should be active.

It was shown that insulin immobilized on various water-insoluble matrices enhances the growth of anchorage-dependent cells more than the native insulin[5,6]. It was considered that this effect was due to long-lasting signal transduction due to inhibited down-regulation[7]. However, the growth-acceleration effect of immobilized insulin might involve effects arising from chemical modification of the insulin and the generation of a multivalent ligand system. These effects of immobilized insulin have not been thoroughly investigated.

In this chapter, to clarify the effect of chemical modification and the generation of multivalent ligand system, we synthesized insulin-polyoxyethylene or insulin-poly(acrylic acid) conjugates and compared their effects with that of insulin immobilized to a poly(acrylic acid)-grafted polystyrene film.

Materials and Methods

Materials

Insulin (bovine origin, No. I-5500) and tresylated ω -methoxypolyoxyethylene (TMPOE, MW=5,000) were purchased from Sigma Chemical

Co. (St. Louis, MO). ^{125}I-insulin was purchased from Daiichi Chemical Ltd. (Tokyo, Japan). Poly(acrylic acid) (PAA, MW=15,000), acrylic acid (AA), and Clear-sol I were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Polystyrene (PSt) was purchased from Wako Pure Chemical Ltd. (Osaka, Japan) and purified by precipitation in methanol before use.

Preparation of Polyoxyethylene Conjugate

The conjugate was synthesized according to the method previously reported[8].The synthetic scheme is shown in Figure 1a. A phosphate-buffered solution (pH 7.0, 0.5 mL) containing tresylated TMPOE (100 μg) and the same solution (0.5 mL) containing insulin (1 mg) with or without ^{125}I-insulin were mixed and stirred at 4 ℃ for 24 h. The product was purified by ultrafiltration (Millipore Molecut II, filtration off below 10 kDa) and referred to as Ins-POE. The formation of the insulin conjugate was confirmed by HPLC using a column packed with Cosmosil 5Diol-120, purchased from Nacalai Tesque, Inc. (Kyoto, Japan) [eluent, 0.02 M phosphate buffer (pH 7.0) + 0.2 M NaCl; elution rate, 1.0 mL/min at room temperature; detection, absorbance at 280 nm].

Preparation of Poly(acrylic acid) Conjugate

The synthetic scheme is shown in Figure 1b. A phosphate-buffered solution (pH 7.0, 0.5 mL) containing PAA (100 μg) was mixed with the same solution (0.5 mL) containing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (water-soluble carbodiimide, WSC, 1 mg) and the mixture was stirred at 4 ℃ for 24 h. The solution containing activated PAA was quickly ultrafiltrated (Millipore Molecut II, filtration off below 10 kDa), mixed with a phosphate-buffered solution (pH 7.0, 1 mL) containing insulin (1 mg) with or without ^{125}I-insulin, then stirred at 4 ℃ for 24 h. Thereafter, a phosphate-buffered solution containing glycine (10 mg, 1 mL) was added to the reaction mixture and stirred at 4 ℃ for a further 6 h to block any activated carboxylic groups of PAA that remained unreacted. The product was purified by ultrafiltration and is referred to as Ins-PAA. The formation of Ins-PAA was confirmed by HPLC as described above for Ins-POE.

Immobilization of Insulin onto a Poly(acrylic acid)-Grafted Polystyrene Film

The synthetic scheme is shown in Figure 1c. A PSt film was cast from a toluene solution (15 wt%) on a circular glass plate (diameter, 15 mm). The film was glow-discharged (200 w) with a high-frequency wave generator (Nihon Koshuha Co.,

Yokohama, Japan) for 20 s under a pressure of 5.65 Pa. The film was exposed to air and incubated in a solution of AA (20 wt%) at 60 ℃ for 24 h. The PAA-grafted PSt film was washed with distilled water until the absence of unreacted monomer and unbound PAA in the washing solution was confirmed by a pH monitor.

The PAA-grafted PSt film was immersed in a phosphate-buffered solution (pH 7.0) containing WSC (1 mg/mL) and ^{125}I-insulin of various concentrations at 4 ℃ for 48 h. After immobilization, the film was repeatedly washed with phosphate-buffered saline (PBS, pH 7.4, NaCl; 8.0 g, KCl; 0.2 g, KH_2PO_4; 0.2 g, $NaHPO_4$; 1.15 g; Mg Cl_2; 0.1 g; $CaCl_2$; 0.1 g in 1 L of distilled water) until the absence of ^{125}I-insulin in the washing solution was confirmed. Non-radiolabeled insulin was immobilized under the same condition. The immobilized insulin is referred to as Ins-PSt. Insulin-adsorbed PSt film was prepared in a similar manner without using WSC. These films were disinfected with 70% ethanol, and washed with sterilized PBS for biological experiments.

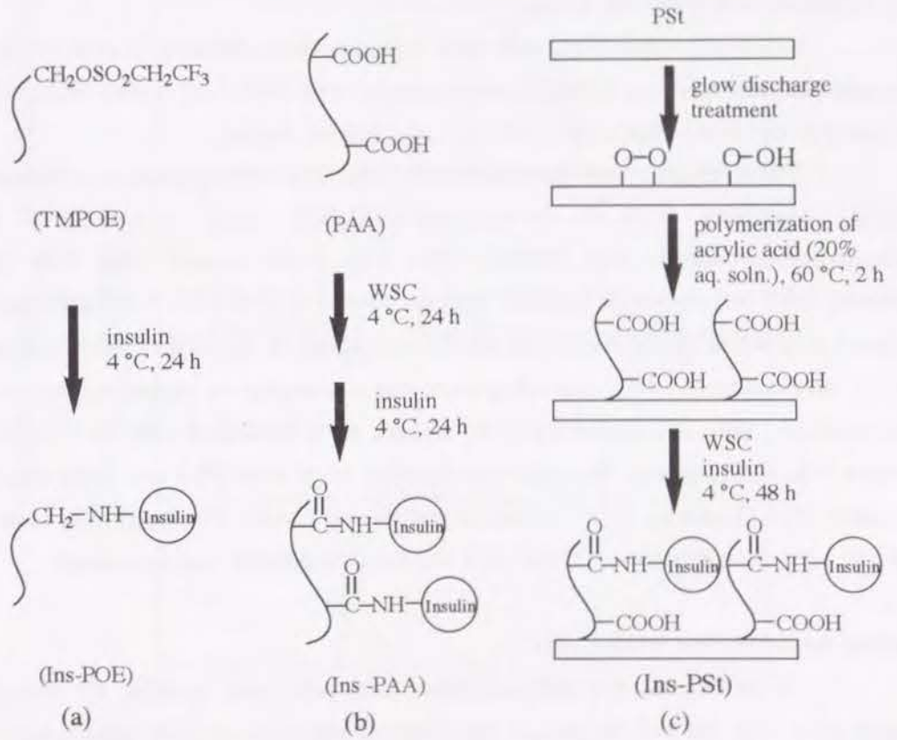

Figure 1 Synthetic schemes of insulin conjugates with (a) polyoxyethylene (POE), (b) poly(acrylic acid) (PAA), and (c) poly(acrylic acid)-grafted polystyrene (PSt) film. WSC, water-soluble carbodiimide.

Measurement of Contact Angle, Electron Spectroscopy for Chemical Analysis (ESCA), Surface Concentration of Carboxylic Acid, and Composition of Conjugates

The contact angle of an air bubble placed on a polystyrene film in water was measured to assess the surface wettability. Prior to the measurement, the PSt film was incubated at 37 ℃ for 24 h. Surface modification of PSt film was analyzed by ESCA using a Perkin Elmer 5500 series Multi-Technique on freeze-dried samples. The surface amount of carboxylic acid was measured using Rhodamine 6G as reported[5].

The composition of insulin in the conjugates was determined by measuring ^{125}I and fluorescence. The labeled conjugates were prepared by the same methods as the non-labeled conjugates. The fluorescence was measured at 345 nm by excitation at 280 nm. The insulin contents were calculated by comparing the radioactivity and fluorescence intensity of insulin conjugates with that of insulin per unit weight.

Cell Culture and Growth Assay

Mouse fibroblast STO cells were subcultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan).

The STO cells were harvested using PBS without magnesium or calcium ions [PBS(-)] containing 0.15 % (w/v) trypsin (2,000 unit/g) and 0.02 % (w/v) ethylenediaminetetraacetic acid (EDTA). The cells were washed once with DMEM containing FBS and once with DMEM, then suspended in DMEM (2×10^4 cells/well) and incubated in 24-well plates containing insulin conjugates in DMEM (1 mL/well) under a 5% CO_2 atmosphere at 37 ℃. The cell growth was determined by measuring the uptake of ^{3}H-thymidine[9]. After cell culture for 45 h, the cells were incubated with ^{3}H-thymidine for a further 3 h. Subsequently the cells were washed once with PBS and three times with ice-cooled TCA (10 wt%), then solubilized with 0.5 N NaOH. The lysate was neutralized with 5 N HCl, then mixed with Clear-sol I and the radioactivity was measured.

Insulin Association with Cells

To determine the internalization into cells and specific or nonspecific complexation with the cell surface of the insulin conjugates (insulin association), STO cells were cultured in the presence of ^{125}I-labeled insulin conjugates for 3, 24 , and 48 h. After washing with PBS(-) for three times, the cells were detached from culture plates using PBS(-) containing 0.02 wt% EDTA until cells attached to the plates became undetectable by optical microscopy. The cells were collected by centrifugation and the

radioactivity was measured by a γ-counter.

Results and Discussion

Synthesis of Water-Soluble Insulin Conjugates

Figure 2 shows the HPLC elution profile of the native insulin and the insulin conjugates with POE and PAA. Ins-POE eluted faster than the native insulin or POE. The

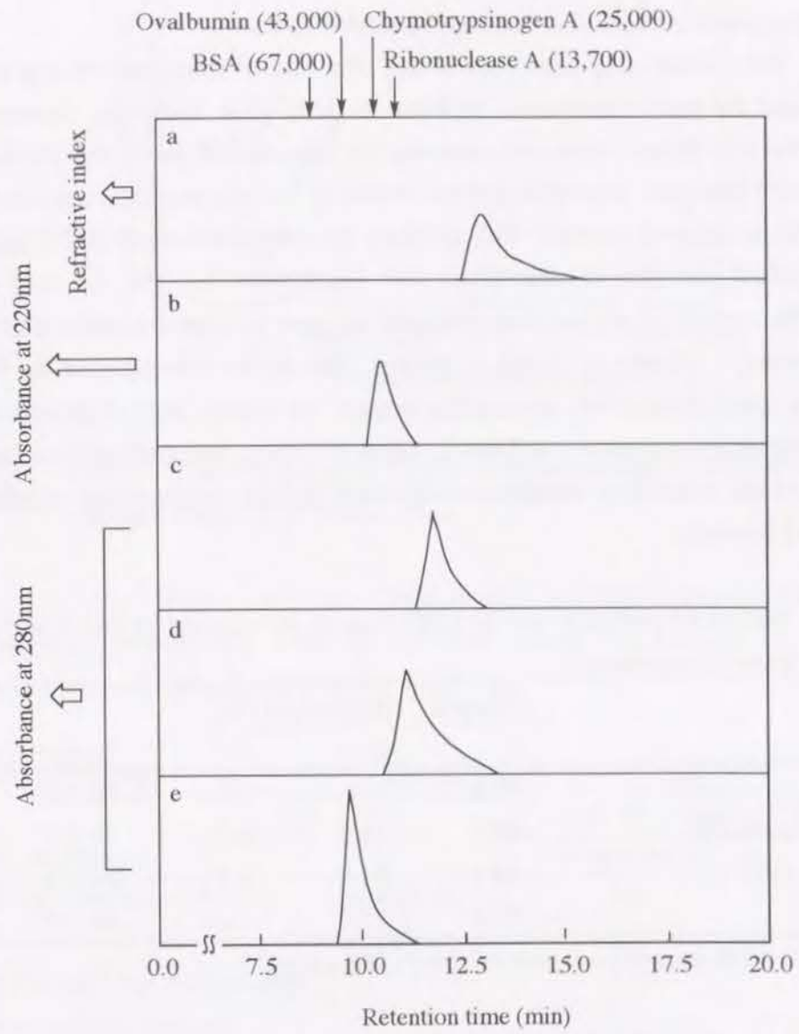

Figure 2 HPLC elution profiles of (a) POE, (b) PAA, (c) native insulin, (d) Ins-POE, and (e) Ins-PAA. BSA, bovine serum albumin. The numbers in parentheses indicate the molecular weight.

Ins-PAA conjugate eluted faster than native insulin or PAA. By measuring the ^{125}I-labeled insulin, it was shown that the insulin contents in Ins-POE and Ins-PAA conjugates were 0.95 ± 0.09 and 4.06 ± 0.15, respectively (± standard deviation, n=5). Fluorescence measurement indicated that the insulin contents in Ins-POE and Ins-PAA conjugates were 0.98 ± 0.11 and 3.90 ± 0.22, respectively (± standard deviation, n = 3). These results demonstrated that Ins-POE was composed of about one insulin molecule and one POE chain, and that Ins-PAA contained about four insulin molecules in one PAA chain.

Graft Copolymerization and Insulin Immobilization

The surface composition of PSt film after various manipulations was analyzed by ESCA and the results are shown in Table 1. After glow-discharge, the content of oxygen in the film surface increased, showing the formation of peroxides, alcohols and other oxidation products. After graft polymerization of AA, the oxygen content increased, indicating the presence of PAA (O, 40% in PAA). The concentration of COOH groups on the PAA-grafted PSt film as determined with Rhodamine 6G was 4.3 ± 0.2× 10^{-8} mol/cm^2. The content of oxygen decreased and nitrogen became detectable after insulin immobilization (N, 15.4%; O, 18.4% in insulin). The surface wettability of the PSt film after various manipulations was estimated in terms of the contact angle of an air bubble in water and the results are shown in Table 1. Glow-discharge and graft polymerization of AA increased the wettability. Insulin immobilization slightly decreased the wettability of PAA-grafted PSt film.

Table 1 Surface properties of modified PSt films as investigated by ESCA and contact angle measurement.

	Element composition (%)			
Sample	C	O	N	Contact angle
PSt	97.8	2.2	0.0	88±3°
Glow-discharged PSt	88.7	11.3	0.0	42±3°
PAA-grafted PSt	78.1	21.9	0.0	35±3°
Ins-PSt※	73.3	15.7	11.0	38±2°

※PAA-grafted PSt immobilized with insulin (0.684 μg/cm^2).

PAA-grafted PSt film was immersed in an aqueous solution of insulin in the presence or absence of WSC. The film was then repeatedly washed with PBS until insulin release from the film was undetectable. Complete removal of insulin, which was weakly adsorbed on the film, required over 20 washes. The amount of insulin

immobilized or adsorbed on the PAA-grafted PSt film was measured using raiolabeled insulin and the results are shown in Figure 3. With increasing feed concentration, the amount of immobilized insulin increased. The maximal surface concentration of immobilized insulin corresponded to complete coverage of the film surface with insulin. A small amount of non-covalently (physically) adsorbed insulin was found on the film surface after repeated washing with PBS.

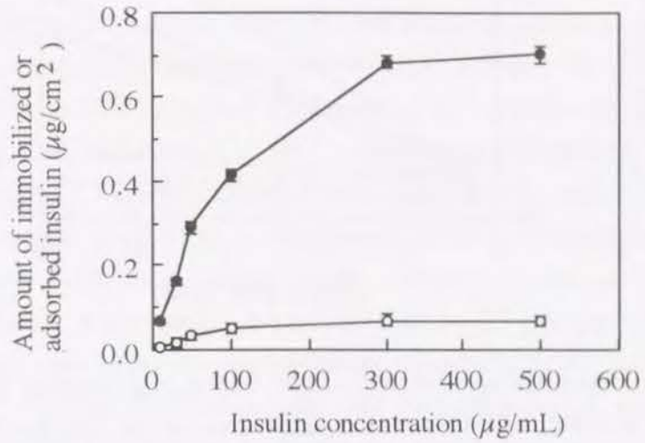

Figure 3 Amount of insulin adsorbed (○) or immobilized (●) on PAA-PSt film. Bars represent standard deviation. n = 6.

Table 2 shows that the covalently immobilized insulin was not released under any conditions investigated, while the adsorbed insulin was released into the culture medium or taken up by cells under some conditions.

Table 2 Percentage of insulin released during 48 h incubation under various conditions

		State of insulin on PSt film	
Culture condition		Immobilized	Adsorbed
Serum-free medium with cells	in medium	0.0	3.3±2.0
	in cells	0.0	26.6±6.0
Serum-free medium without cells		0.0	1.3±0.6
Serum-containing medium		0.0	10.0±1.3
PBS		0.0	0.0

The association of insulin conjugates with cells during culture is shown in Figure 4. Insulin internalization was slightly suppressed by binding to POE, but enhanced

by binding to PAA. On the other hand, Ins-PSt was completely free from insulin internalization, while insulin non-covalently adsorbed on PAA-grafted PSt film was very slowly taken up by the cells.

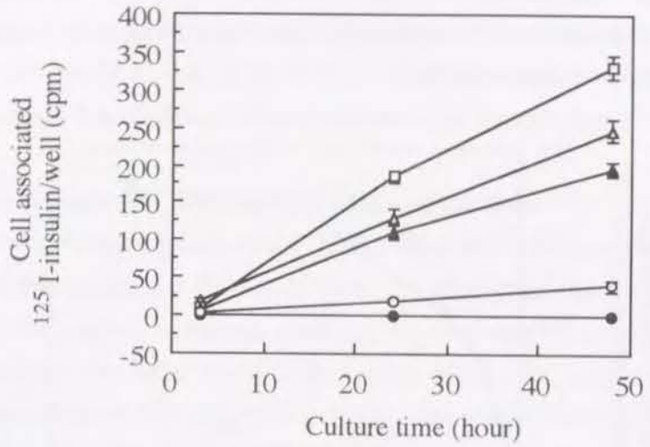

Figure 4 Time-dependent association of insulin with mouse fibroblast STO cells during culture in the presence of (△) native insulin, (▲) Ins-POE, (□) Ins-PAA, (●) Ins-PSt, and (○) insulin adsorbed on PAA-PSt. Bars represent standard deviation. n = 6.

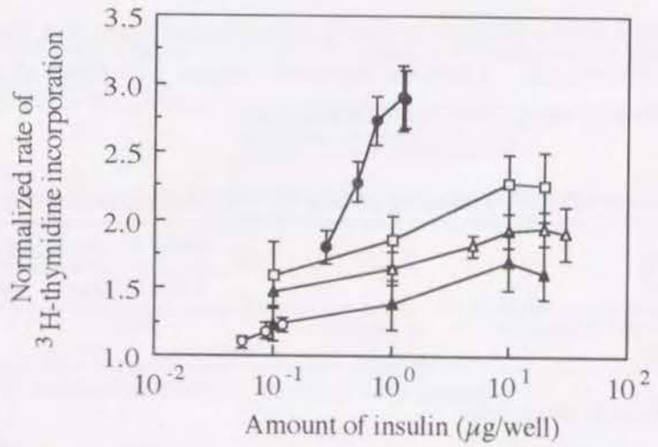

Figure 5 Growth rate of mouse fibroblast STO cells in the presence of (△) native insulin, (▲) Ins-POE, (□) Ins-PAA, (●) Ins-PSt, and (○) insulin adsorbed on PAA-PSt. Bars represent standard deviation. n=6.

Mitogenic Activities of Insulin Conjugates

Figure 5 shows the rate of DNA synthesis in mouse fibrobast STO cells cultured in the presence of various insulin conjugates. The mitogenic effect of Ins-POE was lower than that of native insulin. The effect of Ins-PAA was a little higher than that of the native insulin. Small amounts of Ins-PSt (1/10 to 1/20 fold the amount of native insulin) stimulated DNA synthesis in STO cells. The maximal mitogenic effect of Ins-PSt was much greater than that of the water-soluble insulin conjugates investigated.

Insulin has two chains, A and B, and three free amino groups, A1-glycine, B1-phenylalanine, and B29-lysine. The reactivity is A1 > B29 > B1. The N-terminus of the A-chain is the most reactive and is considered to be in the neighborhood of the receptor binding site[10]. Markussen *et al.*[11] coupled insulin selectively through the B1 amino group to divinyl sulfone-activated agarose using insulin, which was protected in positions A1 and B29, to construct a high capacity affinity chromatography system for insulin receptors. On the other hand, Schoelson *et al.*[12] incorporated benzoylphenylalanine, a photoactive amino acid into the 25 position of the B-chain by chemical synthesis and enzymatic semisynthesis to investigate the interaction between insulin and the receptor. They concluded that the efficiency of cross-linking was unusually high, ranging from 60 to 100% and the cross-linking resulted in receptor and kinase activation. Weiland *et al.*[13] synthesized a covalently-dimerized insulin derivative B29,B29'-suberoyl-insulin and indicated that the derivative competitively inhibited insulin-stimulated DNA synthesis in 3T3-L1 fatty fibroblasts. However, it had no effect on mature rat adipocytes. These results demonstrate that it is difficult to estimate the activity of modified insulin.

In this study, the mitogenic effect of insulin was reduced by coupling to POE. It was considered that POE molecule hindered access of the insulin to the receptor, because insulin is linked to POE mainly through the chemically reactive and biologically active sites, A1 and B29. However, the Ins-PAA conjugate apparently possessed higher mitogenic activity than the Ins-POE conjugate, although it was also considered that insulin is linked to PAA through A1 and B29. This should be due to multivalency of Ins-PAA. The Ins-PAA conjugate can be active by enhancing receptor dimerization, as well as the aggregation of insulin conjugate/receptor complex.

Covalent immobilization by insulin onto polymer matrix was first reported by Cuatrecasas in 1969, who found that the immobilized insulin stimulates isolated fat cells[14]. However, Oka and Topper found that some material with insulin-like activity was released from insulin-Sepharose[15]. This substance had a greater activity than native insulin and was referred to as super-active insulin. Wilchek and coworkers [16] considered

that the biological effects attributed to the insulin-Sepharose conjugate may be best explained in terms of insulin/serum protein-substituted guanidine released from the Sepharose matrix during cell culture.

The present study showed that immobilized insulin, Ins-PSt was more mitogenic than Ins-PAA. In addition to multivalency, some other mechanisms must be operating in the action of Ins-PSt. Ito *et al.*[7] reported that insulin immobilized on surface-hydrolyzed poly(methyl methacrylate) film activated insulin receptors longer than native insulin, due to inhibited down-regulation. Ins-PSt should also activate the cellular signal-transduction system over a long period without internalization. Membrane-anchored growth factors or cytokines regulate cells as "juxtacrine stimulators"[17]. Immobilized insulin is considered to be "an artificial juxtacrine stimulator". The high activity of the artificial system will extend the concept of juxtacrine stimulation.

Proteins physically adsorb onto solid materials and it is impossible to completely remove them by washing[18,19]. Therefore, some non-covalently adsorbed insulin remained on PAA-grafted PSt film even after repeated washing. However, the amount was very small, it was slowly released from the surface, and the mitogenic effect was negligible. In Ins-PSt, insulin was covalently bound and none was released during cell culture. There should be no space where insulin could physically adsorb on the activated PAA-grafted PSt film surface.

Another problem concerning the insulin-Sepharose gel conjugate is the porosity or the rough surface of the matrix, which hinders access of cells to immobilized insulin. Horwitz and coworkers[4] pointed out the importance of a smooth surface in the activity of immobilized biosignal proteins, and reported that interleukin-2 immobilized to plasma-treated polystyrene film maintained the viability of an interleukin-2 dependent-cell line.

In the present investigation, the effectiveness of insulin immobilization onto insoluble matrix was demonstrated by comparing Ins-PSt with Ins-PAA or Ins-POE conjugates. The effectiveness probably arose from inhibited internalization. A comparison of two types of immobilization matrices, PSt film and Sepharose gel, indicated that the smooth film surface induced efficient interaction of immobilized insulin with adsorbed cells. The contribution of the lack of internalization to the activity of immobilized biosignal proteins was quantified by using smooth PSt film.

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Chapter 2

Photo-Immobilization of Insulin onto Polystyrene Plate for Protein-Free Cell Culture

Introduction

Mammalian cell culture is important in terms of both biological research and industrial applications. To support cell growth, serum, in addition to nutrients, is necessary. However, serum is expensive and it contains unknown materials. Much effort has been directed toward cell culture without serum in the presence of protein growth factors. Also, natural substrata or artificial biomaterials in cell culture and tissue engineering have been advocated[1,2].

It was shown that insulin immobilized on water-insoluble matrix enhances the growth of anchorage-dependent cells to a greater extent than the native insulin as shown in chapter 1. However, the choice of matrices was limited because of the need for surface functional groups for protein immobilization. In order to achieve covalent bonding between a protein such as cell growth factors and a polymer surface, the currently available methods use condensation reactions at water/polymer interface[3-13]. In these cases, active hydrogen-containing functional groups such as amino, carboxyl, hydroxyl, or mercapto groups, which may be derived from surface hydrolysis and surface graft polymerization, must exist in the outermost layer of polymer surfaces for chemical bonds with proteins. In this chapter, a method for facile preparation of immobilized insulin was devised. The mitogenic activity of immobilized insulin was investigated.

Materials and Methods

Materials

Microscint™-20 was a product of the Canberra Company (Groningan, The Netherlands). Trichloroacetic acid (TCA) was purchased from Nacalai Tesque (Kyoto, Japan). *N,N*-Dimethylformamide (DMF), and acetonitrile were purchased from Wako Pure Chemical. Ltd. (Osaka, Japan). Dicyclohexylcarbodiimide (DCC) and 4-azidobenzoic acid were purchased from Tokyo Kasei Co. (Tokyo, Japan). *N*-Hydroxysuccinimide was purchased from the Protein Institute Inc. (Minoh, Japan).

Tissue culture PSt plate with 24 wells (Sumilon) was purchased from Akita Sumitomo Bake Co. (Akita, Japan).

Preparation of Photo-Immobilized Insulin

The synthetic scheme of photo-immobilized insulin is shown in Figure 1. Insulin coupled with 4-azidobenzoyloxy was dissolved in water and added dropwise to the wells of a PSt plate. The water was evaporated in air, and then the wells were irradiated with UV light.

N-(4-Azidobenzoyloxy)succinimide was prepared as reported by Matsuda and Sugawara[14]. A solution of DCC (13.3 g, 64.6 mmol) in tetrahydrofuran (THF, 50 mL) was added dropwise to a solution of *N*-hydroxysuccinimide (7.43 g, 64.6 mmol) and 4-azidobenzoic acid (9.57 g, 58.7 mmol) in THF (150 mL) in an ice bath under stirring. After 3 h, the reaction mixture was warmed slowly to room temperature and stirring was continued overnight. The white solid that formed was filtered off, and the solvent was removed under reduced pressure. The remained yellow residue was crystallized from isopropyl alcohol/isopropyl ether.

To a solution of *N*-(4-azidobenzoyloxy)succinimide of various concentrations in DMF and PBS (pH 7.4) (DMF/PBS = 4/1; 10 mL) was added insulin (2.0 mg, 0.33 μmol) with stirring in an ice bath. The solution was stirred at 4 ℃ for 48 h. The product was purified by ultrafiltration (Millipore MoleCut II, filtration off below 3 kDa). The content of incorporated azidobenzoyl groups per unit weight of insulin derivative was determined by ultraviolet absorption and fluorescence intensity. The amount of azidobenzoic acid and insulin were calibrated by measuring the ultraviolet absorption at 280 nm and fluorescence intensity at 345 nm by excitation at 280 nm, respectively.

An aqueous solution of various concentrations of azidophenyl-derivatized insulin (0.1 mL) was placed in each of the 24 wells (diameter = 18 mm) of a PSt tissue culture plate. After air-drying, the wells were irradiated for 10 s using a UV lamp (Koala 25, 100V) from a distance of 5 cm. Thereafter, the plate was washed repeatedly with PBS. The effectiveness of the wash was confirmed by using ^{125}I-insulin immobilized to a plate as described in the following. The plate was disinfected with 70% (v/v) ethanol and washed with sterilized PBS for biological experiments.

Azidophenyl-derivatized ^{125}I-insulin was immobilized onto the wells of a PSt plate by photo-irradiation. The plate was then washed repeatedly with PBS until the release of ^{125}I-insulin in the washing solution became undetectable. Thereafter, Microscint™-20 (1 mL) was added to the ^{125}I-insulin-immobilized well and the radioactivity level was measured.

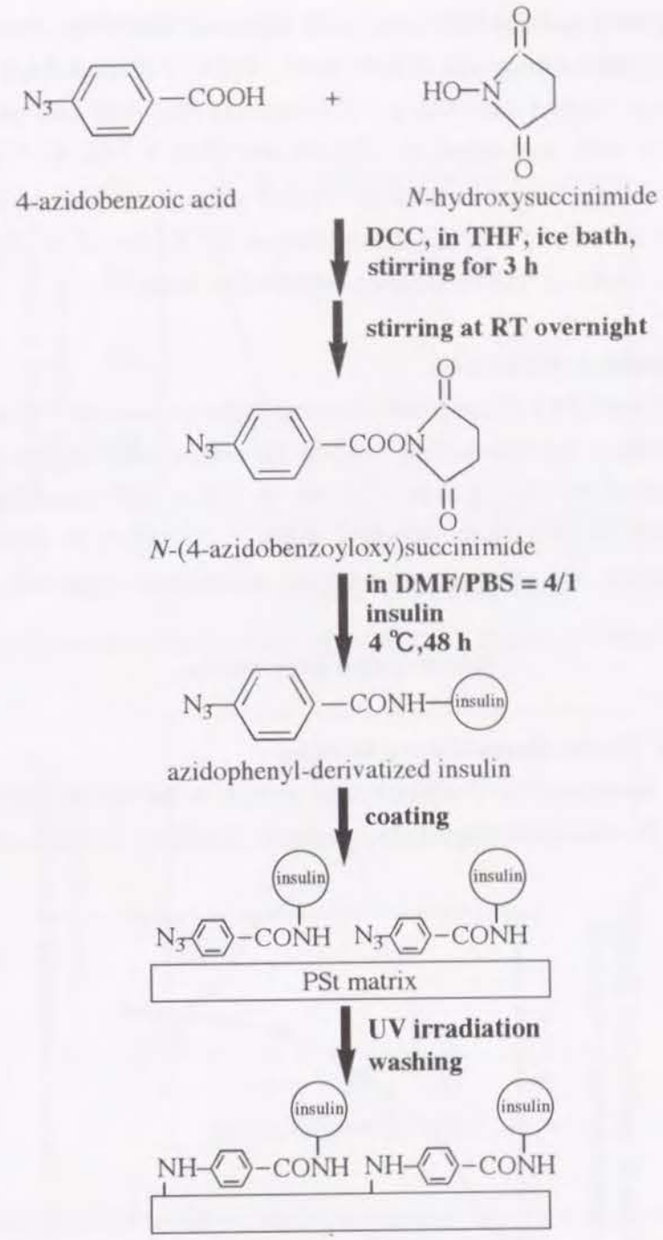


Figure 1 Preparative scheme of photo-immobilized insulin.

Cell Culture and Growth Assay

Mouse fibroblast STO and Chinese hamster ovary CHO-K1 cells were subcultured in DMEM with 10% (v/v) FBS and Ham's F12 medium containing 10% (v/v) FBS, respectively. The media were purchased from Nissui Pharmaceutical Co. Ltd..

The STO and CHO-K1 cells were harvested with PBS(-) containing 0.15% (w/v) trypsin (2,000 units/g) and 0.02% (w/v) EDTA. STO and CHO-K1 cells were washed once with DMEM and Ham's F12 containing FBS and once with DMEM and Ham's F12 and then suspended in DMEM and Ham's F12 (2×10^4 cells/well), respectively. The cells were incubated in 24-well polystyrene plate in the presence of insulin derivatives under a 5% CO_2 atmosphere at 37 ℃ for 48 h. Cell growth was estimated by the uptake of ^{3}H-thymidine as described in chapter 1.

Insulin Association with Cells

STO and CHO-K1 cells were cultured in the presence of ^{125}I-labeled insulin. After 48 h of culture, the radioactivity level in the culture medium was measured. The cells were collected by rinsing with 0.02 wt % EDTA and centrifugation, and the radioactivity levels in cells were measured using a γ-counter to determine the total amount of insulin that was either incorporated into or adsorbed on the cells.

Results and Discussion

Preparation of Photo-Immobilized Insulin

The incorporation of azidobenzoyl groups in the insulin increased with the concentration of *N*-(4-azidobenzoyloxy)succinimide (Figure 2). Insulin contains three

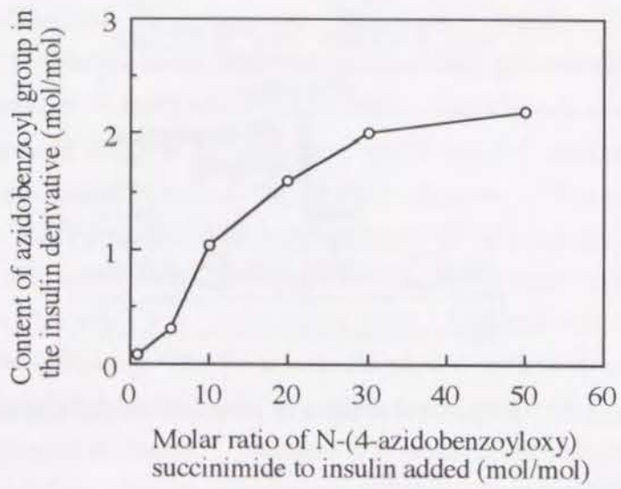


Figure 2 Content of the azidobenzoyl group in the insulin derivative *versus* the concentration of *N*-(4-azidobenzoyloxy)succinimide.

free amino groups, referred to as A1, B1, and B29. In this study, the derivative containing 1.04 azidophenyl groups per molecule of insulin was used for photo-immobilization.

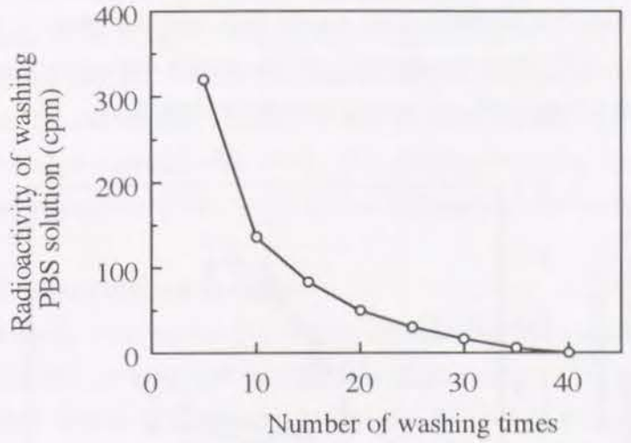


Figure 3 Radioactivity in the washing solution of ^{125}I-insulin-immobilized matrix

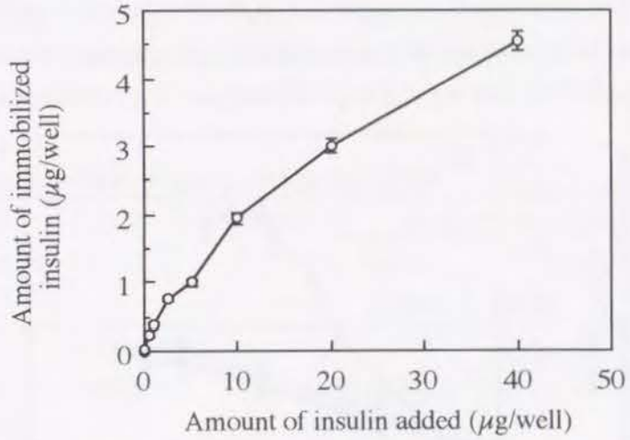
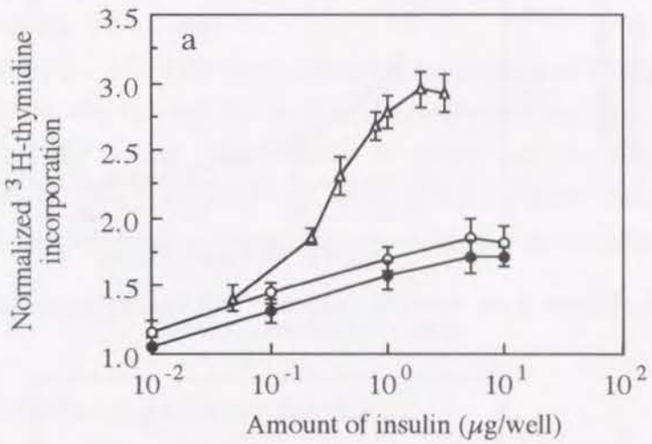


Figure 4 Amount of immobilized insulin as calculated from the amount of radioactivity of ^{125}I-insulin *versus* the amount of the coated insulin derivative. Bars represent the standard deviations, n = 6.

The insulin derivative was dissolved in water and dropped into 24 wells of a polystyrene plate. The matrix was dried in air and photo-irradiated for 10 s. When culturing cells on immobilized insulin, the absence of leakage should be confirmed[6,15].

Therefore, the insulin-immobilized matrix was washed until insulin leakage was absent (Figure 3).

The amount of photo-immobilized insulin increased with the amount of the insulin derivative coated on the matrix (Figure 4). By assuming a monolayer formation, the calculated surface concentration of the insulin derivative was about 0.18 - 0.34 μg/cm^2 (0.46 - 0.86 μg/well)[16]. The concentration of the insulin derivative immobilized on the PSt plate exceeded 4.5 μg/well, suggesting multilayer formation.



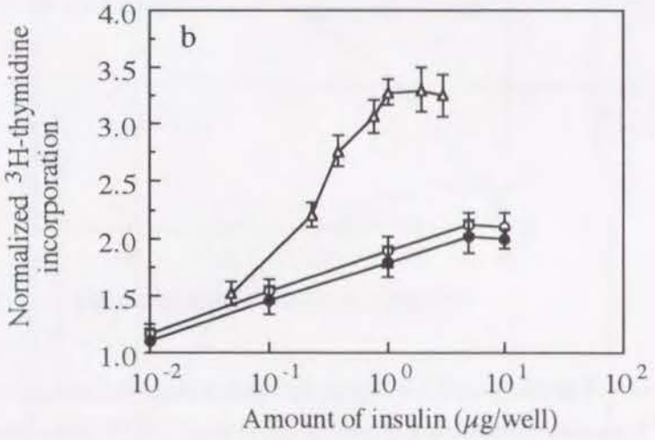


Figure 5 Growth of Chinese hamster ovary CHO-K1 (a) and mouse fibroblast STO cells (b) in the presence of (○) native, (●) azidophenyl-derivatized and (△) photo-immobilized insulin. The amount of ^{3}H-thymidine incorporated in cells cultured on tissue culture polystyrene plate in the absence of insulin or insulin derivatives was taken as 1.0. Bars represent the standard deviations, n = 6.

Cell Growth in the Presence of Photo-Immobilized Insulin

Mouse fibroblast STO and Chinese hamster ovary CHO-K1 cells were cultured in the presence of various types of insulin derivatives (Figure 5). The incorporation of azidobenzoyl groups into insulin slightly reduced its mitogenic activity. On the other hand, small amounts of immobilized insulin (1/10 - 1/20 fold the amount of the native insulin or the azidophenyl-derivatized insulin) enhanced the growth of these cells. In addition, the maximal mitogenic effect of the immobilized insulin was higher than that of the water-soluble insulin derivatives. The growth-enhancing effect tended to level off at concentrations corresponding to monolayer formation on the surface.

Fate of Photo-Immobilized Insulin

The study with radioactive insulin showed that the native and azidophenyl-derivatized insulin were internalized into the cells during culture (Table 1). However, the photo-immobilized insulin was neither released into the culture medium nor internalized into the cells. Ito *et al.*[6] have reported that insulin immobilized onto a surface-hydrolyzed poly(methyl methacrylate) film stimulates the insulin receptor and the related signal transduction proteins for a longer period than native insulin by inhibiting down-regulation of the insulin receptors caused by internalization. The photo-immobilized insulin should also stimulate the systems for a long period to account for the high mitogenic activity.

Table 1 Fate of insulin derivatives during cell culture.

Sample	Cell	Radioactivity (cpm/well)[1]		
		Before culture	After culture	
			In cell	In medium
Native insulin[2]	CHO-K1	10690	396±25	10253±81
	STO	10690	422±17	10268±96
Azidophenyl-derivatized insulin[2]	CHO-K1	10690	378±19	10287±108
	STO	10690	412±8	10278±73
Photo-immobilized insulin[3]	CHO-K1	10743	0	0
	STO	10743	0	0

1. The average value and the standard deviation, n = 3.
2. Amount of insulin was 1.000 μg/well.
3. Amount of insulin immobilized was 1.005 μg/well.

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Chapter 3

Enhancement of Mitogenic Effect by Artificial Juxtacrine Signaling from Photo-Immobilized Epidermal Growth Factor

Introduction

Chemical modification of biological signaling molecules such as hormones and growth factors, with a soluble or insoluble supporting matrix is important for elucidating the sites and mechanisms of signal transduction. Signaling molecules exert diverse biological effects by binding and activating cell surface receptors, which, in turn, activate intracellular signaling pathways. However, the interaction of conjugated hormones and growth factors with cells is poorly understood.

It was shown that insulin immobilized on surface-modified matrices enhances the growth of anchorage-dependent cells to a greater extent than native insulin as shown in chapters 1 and 2. It was also demonstrated that, although more time was required for immobilized insulin to stimulate insulin receptors on Chinese hamster ovary (CHO) cells than that for native insulin, receptor activation persisted for longer period with immobilized insulin[1].

Epidermal growth factor (EGF) is a member of a family of growth factors that are derived from membrane-anchored, biologically active precursors[2,3]. These precursors activate cognate receptors on adjacent cells, and communicate by "juxtacrine stimulation". These mechanisms were deduced from studies of intercellular regulation by paraformaldehyde-fixed cells that express the growth factor[4].

In this chapter, EGF was photo-immobilized onto a polystyrene plate by the same method as described in chapter 2, and the mitogenic activity was investigated.

Materials and Methods

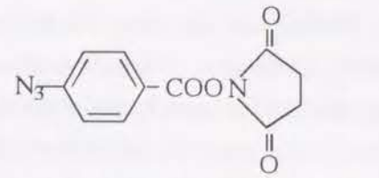
Materials

Mouse EGF was purchased from Toyobo (Osaka, Japan). ^{125}I-EGF was purchased from Daiichi Chem. Ltd. (Tokyo, Japan). The cell counting kit containing 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate was purchased from Dojin Chem. (Kumamoto, Japan).

Preparation of Photo-Reactive EGF

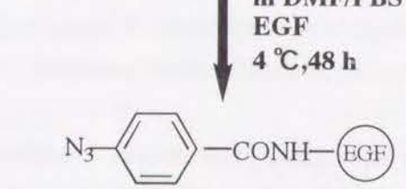
EGF (0.1 mg, 16.5 nmol) was added to a solution of *N*-(4-azidobenzoyloxy)succinimide (0.43 mg, 1.65 μmol) which was prepared as described in chapter 2 in DMF and PBS (pH 7.4) (DMF/PBS = 4/1; 1 mL) with stirring in an ice bath. The solution was continuously stirred at 4℃ for 72 h, then ultrafiltrated (Millipore MoleCut II, filtration off below 3 kDa).

The product was analyzed by HPLC [column, Cosmosil 5C18-AR purchased from Nacalai Tesque (Kyoto, Japan); eluent, a linear gradienet from 0.05% trifluoroacetic acid (TFA)/20% acetonitrile to 0.05% TFA/60% acetonitrile; elution rate, 1.0 ml/min for 20 min; temperature, 30℃; detection, absorbance at 280 nm].



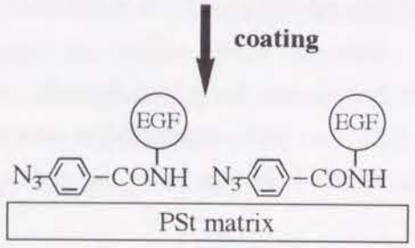
N-(4-azidobenzoyloxy)succinimide

in DMF/PBS = 4/1
EGF
4 ℃,48 h



azidophenyl-derivatized EGF

coating



UV irradiation
washing

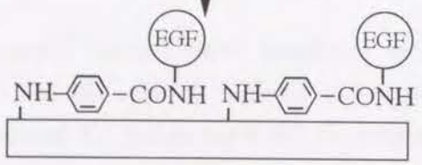


Figure 1 Procedure of photo-immobilization of EGF

Photo-Immobilization of EGF

The photo-immobilization procedure is shown in Fig. 1. Aqueous solutions of various concentrations of the azidophenyl-derivatized EGF (0.1 mL) were added to 24 wells (diameter, 18 mm) of a PSt tissue culture plate. After air-drying, the wells were irradiated for 10 s using a UV lamp (Koala 25, 100V) from a distance of 5 cm. Thereafter, the plate was repeatedly washed with PBS. The effectiveness of washing was confirmed by using ^{125}I-EGF. The 24-well plate was disinfected with 70% (v/v) ethanol, and washed with sterilized PBS for biological experiments.

Azidophenyl-derivatized ^{125}I-EGF was prepared and immobilized onto a PSt plate by photo-irradiation under the same conditions described above. Thereafter, the plate was repeatedly washed with PBS until the release of ^{125}I-EGF in the washing solution became undetectable. Microscint™-20 (1 mL) was then added to the ^{125}I-EGF-immobilized wells and the radioactivity levels were measured.

Cell Culture and Growth Assay

Mouse hybridoma Tg1-1HMS cells were subcultured in PM-1000 medium with 0.2% (v/v) FBS. The medium was provided by Eiken Chem. (Tokyo, Japan).

The STO and CHO-K1 cells, which were subcultured and collected as described in chapter 2, were suspended in DMEM and Ham's F12 medium (2×10^4 cells/well, 1mL), respectively, and incubated in 24-well polystyrene plate in the presence of EGF derivatives under a 5% CO_2 atmosphere at 37 ℃ for 48 h. Cell growth was estimated by the amount of ^{3}H-thymidine uptake.

Tg1-1HMS cells were harvested from a suspension culture flask by centrifugation. After washing once with PM-1000 medium, they were suspended in PM-1000 medium, then incubated in 24-well PSt plate (2×10^4 cells/well, 1 mL) in the presence of EGF derivatives under a 5% CO_2 atmosphere at 37 ℃ for 48 h. The cell number was photometrically estimated using the cell counting kit as described[5].

EGF Association with Cell

STO, CHO-K1 and Tg1-1HMS cells were cultured in the presence of ^{125}I-labeled EGF as described above. After 48 h, the cells were collected by centrifugation after rinsing with 0.02 wt% EDTA solution. The radioactivity levels in the culture media or in the cells were measured.

Results and Discussion

Photo-Immobilized EGF Conjugate

Figure 2 shows the elution profiles of 4-azidobenzoic acid, *N*-(4-azidobenzoyloxy)succinimide, EGF and the modified EGF by HPLC. The retention time of *N*-(4-azidobenzoyloxy)-succinimide was longer than that of 4-azidobenzoic acid due to an increase of hydrophobicity. The retention time of the EGF derivative was longer than that of native EGF because of connection with an hydrophobic azidobenzoyl group. EGF has only one amino group in the N-terminus, to which the azidophenyl group was considered to bind.

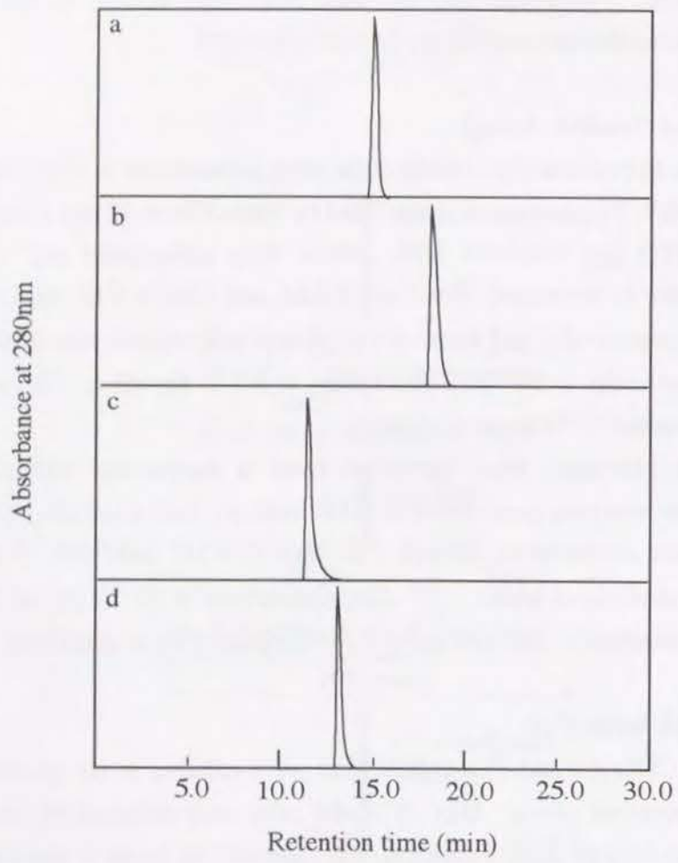


Figure 2 HPLC elution profiles of (a) 4-azidobenzoic acid, (b) *N*-(4-azidobenzoyloxy)-succinimide, (c) native EGF and (d) azidophenyl-derivatized EGF.

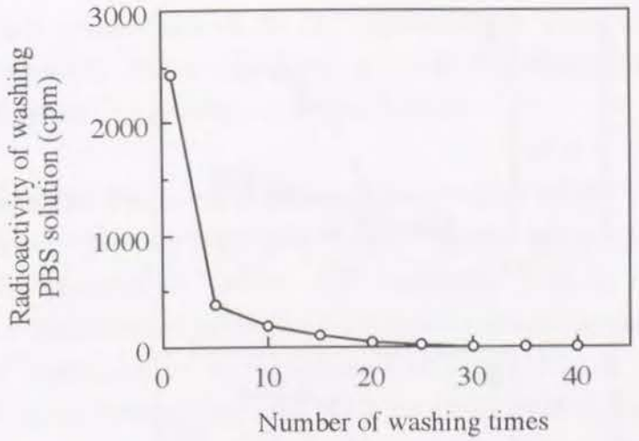


Figure 3 Radioactivity in the washing solution of ^{125}I-EGF- immobilized matrix.

The EGF derivative was dissolved in water and dropped into 24 wells of a PSt plate. The plate was air-dried and photo-irradiated for 10 s. And then, the EGF-immobilized plate was washed at least thirty times until there was no detectable EGF in the washing solution (Figure 3).

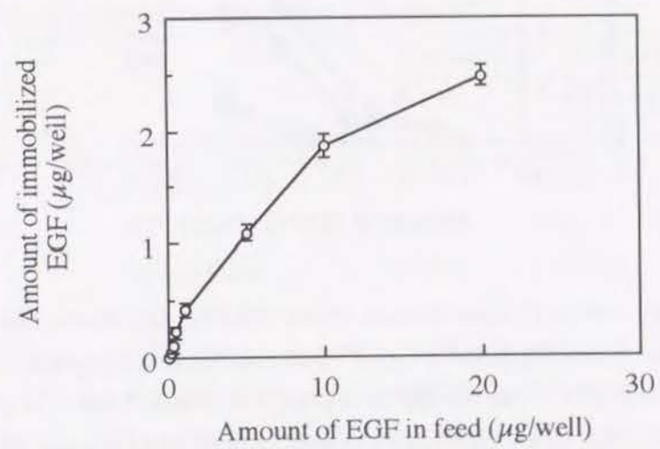


Figure 4 The amount of immobilized EGF calculated from the radioactivity level of ^{125}I-EGF *versus* the amount of coated EGF derivative. Bars represent the standard deviations, n = 6.

The amount of photo-immobilized EGF increased with the amount of EGF derivative coated onto the matrix (Figure 4). Assuming a monolayer formation, the

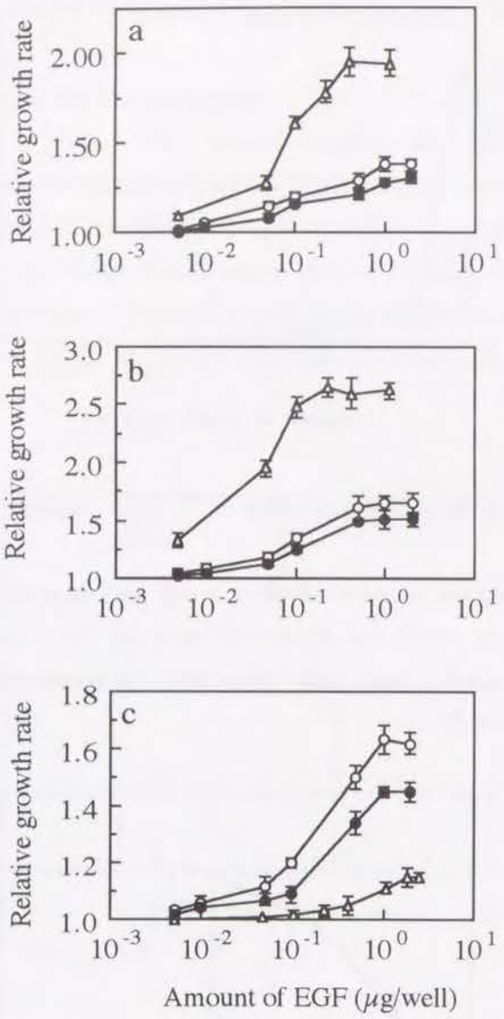


Figure 5 Growth rate of Chinese hamster ovary CHO-K1 (a), mouse fibroblast STO (b) and mouse hybridoma Tg1-1 HMS cells (c) in polystyrene plates in the presence of (○) native, (●) azidophenyl-derivatized and (△) photo-immobilized EGF. The relative growth rates of CHO-K1 and STO cells calculated from the amount of ^{3}H-thymidine incorporated into cells cultured on polystyrene tissue culture plates in the absence of EGF or the EGF derivatives were taken as 1.0. The relative cell growth rate of Tg1-1 HMS cells calculated from the photometric absorbance of medium, in which cells were cultured in the absence of EGF or EGF derivatives, was taken as 1.0. Bars represent the standard deviations, n = 6.

calculated surface concentration of the EGF derivative was about 0.18 - 0.34 μg/cm² (0.46 - 0.86 μg/well). The concentration of the EGF immobilized on the PSt plate exceeded 0.86 μg/well, suggesting a multilayer formation.

Cell Growth in the Presence of Photo-Immobilized EGF

Mouse fibroblast STO and Chinese hamster ovary CHO-K1 cells were cultured in the presence of various EGF derivatives (Figures 5a and 5b). The incorporation of azidobenzoyl group into EGF slightly reduced its mitogenic activity. On the other hand, small amounts of immobilized EGF (1/10 - 1/20 fold the amount of the native EGF or the azidophenyl-derivative EGF) were sufficient to enhance the cell growth. In addition, the maximal mitogenic effect of the immobilized EGF was much higher than that of the water-soluble EGF derivatives. The growth-enhancing effect tended to level off in the region of concentrations corresponding to monolayer formation on the surface.

The photo-immobilized EGF did not enhance the growth of mouse hybridoma cells (Figure 5c). Water-soluble EGFs enhanced the cell growth more than the immobilized EGF. Because the cells were anchorage-independent, there was not enough interaction between the immobilized EGF and the receptor to effect signal transduction.

Table 1 Fate of EGF derivatives during cell culture

Sample	Cell	Radioactivity (cpm/well)[1]		
		Before culture	After cell culture	
			In cell	In medium
Native EGF[2]	CHO-K1	11050	131±18	10896±31
	STO	11050	226±14	10801±46
	Tg1-1HMS	11050	251±31	10791±75
Azidophenyl-Derivatized EGF[2]	CHO-K1	11050	120±33	10897±56
	STO	11050	218±32	10817±27
	Tg1-1HMS	11050	240±50	10795±89
Photo-Immobilized EGF[3]	CHO-K1	10774	0	0
	STO	10774	0	0
	Tg1-1HMS	10774	0	0

1. The average value and the standard deviation, n = 3.
2. Amount of EGF was 500ng/well.
3. Amount of EGF immobilized was 415 ng/well.

Fate of Photo-Immobilized EGF

A study with radioactive EGF showed that the native EGF and the azidophenyl-derivatized EGF were internalized into the cells during culture (Table 1). However, the photo-immobilized EGF was neither released into the culture medium nor internalized into the cells. This lack of internalization probably caused the high mitogenic activity by inhibiting the down-regulation of EGF receptors.

EGF conjugated with fluorescein[6,7], urogastrone[8], ricin A[9-11], *Pseudomonas* endotoxin[11], *Pseudomonas* exotoxin[12], diphtheria toxin[13], β-amanitin-poly-L-ornithine[14], dextran[15-17], or fusion peptides[18] binds to EGF receptors and the conjugates exhibit varying degrees of mitogenic activity. The present study demonstrated that the mitogenic effect of immobilized EGF was greater than that of native EGF and thus smaller amounts of immobilized growth factor were sufficient to enhance cell growth of anchorage-dependent cells. The local concentration of EGF on the surface was sufficient to promote effective interaction with the EGF receptors of adsorbed cells.

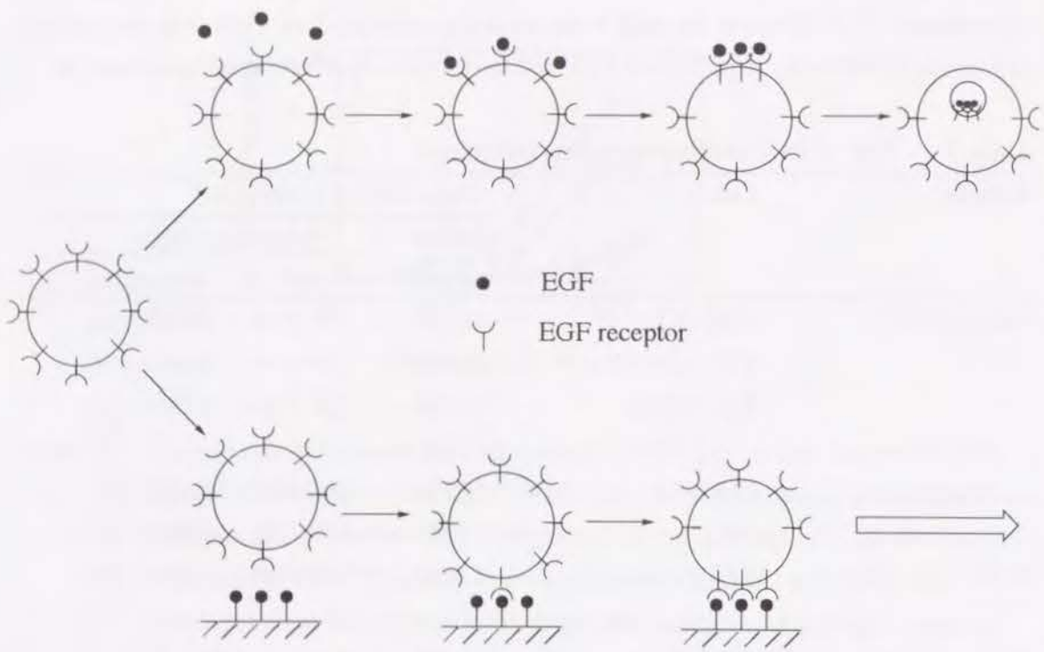


Figure 6 Schematic representation of the interaction between cells and soluble EGF and immobilized EGF. Soluble EGF interacts with the cells to form a complex with the receptor, that is internalized. In contrast, immobilized EGF interacts with the cells for a long period without internalization.

The greater mitogenic effect of photo-immobilized EGF probably results from the fact that the complex of immobilized EGF with the EGF receptors cannot be internalized and therefore, receptors are not down-regulated as shown in Fig. 6. Ito *et al.*[1] concluded this from the finding using immobilized insulin. It is also possible that by preventing the lateral diffusion of activated receptors in the cell membrane, immobilized EGF reduces the opportunity for the receptors to encounter inhibitory regulators such as tyrosine phosphatases or serine/threonine kinases.

Wakshull and Wharton[19] have shown that concanavalin A stabilizes complexes of EGF with its receptor, thus inhibiting receptor endocytosis, and that when the stabilized complex is located at the cell surface, there is an acute biological response to EGF (RNA, but not DNA synthesis). However, cells expressing recombinant internalization-deficient mutant EGF receptors are fully activated by EGF[20-23]. Various native extracellular matrices and adhesion factors also activate intracellular signaling pathways by interacting with integrins, without inducing integrin internalization[24-26].

In addition, EGF stimulates cell growth in the membrane-anchored state, according to the mode of intercellular communication known as juxtacrine stimulation[2,3], EGF is the first polypeptide mitogen to be identified, and the prototype of a large family. Members of the EGF family stimulate EGF receptor phosphorylation, mitogenesis, and Ca^{2+} uptake, which is termed "juxtacrine stimulation". This study demonstrated an artificial juxtacrine stimulation with immobilized EGF. The lack of an effect of photo-immobilized EGF on anchorage-independent cells also demonstrated the importance of direct interaction between cells and the immobilized EGF. This technique will be useful to investigate the signal transduction mechanism and to design a new biomaterial for tissue engineering.

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Chapter 4

Switching from Growth to Differentiation in PC12 Cell by Immobilized Epidermal Growth Factor

Introduction

Signaling by receptors with tyrosine kinase activity plays an important role in the control of diverse cellular processes, such as growth, differentiation, metabolic homeostasis and motility. The binding of polypeptide growth and differentiation factors to receptors that have tyrosine kinase activity promotes receptor dimerization, which is, in turn, responsible for activating the intrinsic tyrosine kinase activity, leading to autophosphorylation of the receptor [1]. The primary response to signaling through certain receptor tyrosine kinases is the stimulation of cell proliferation, whereas activation of other receptor proteins inhibits cell growth and promotes cell differentiation. Moreover, the same receptor tyrosine kinases can either inhibit or stimulate cell proliferation when expressed in different cellular contexts [2-4]. For example, the addition of nerve growth factor (NGF) to phaeochromocytoma PC12 cells or sympathetic neurons leads to neuronal differentiation and cell survival, whereas in the case of fibroblasts, NGF promotes cell proliferation and oncogenesis. The same receptor is involved in signaling from NGF in both cases. The biological response is thus determined both by the properties of receptor tyrosine kinase and by the cellular context in which it is expressed. Furthermore, several recent studies have shown that insulin and epidermal growth factor (EGF) stimulates PC12 cells to cell proliferation, but promote PC12 overexpessing insulin or EGF receptor to neuronal differentiation. The kinetics of mitogen-activated protein (MAP) kinase activation in the two kinds of PC12 cells differs after stimulation by insulin and EGF. Insulin and EGF applied to PC12 cells induce transient stimulation of MAP kinase, but in the case of PC12 overexpessing insulin or EGF receptors, they induce sustained stimulation and nuclear translocation of this enzyme as NGF does[5,6].

Ito *et al.*[7] reported that immobilized EGF activates MAP kinase of Chinese hamster ovary (CHO) cells overexpressing EGF receptors for longer time than native EGF and that the growth of cells was enhanced by the immobilization. EGF belongs to a family of membrane-bound growth factors that activate receptors according to "juxtacrine" mode [8-12]. The stimulation by EGF immobilized on an insoluble matrix could be regarded as an artificial juxtacrine stimulation as shown in chapter 3. In this chapter, PC12 cells

were cultured in the presence of native EGF or immobilized EGF and their effects on PC12 cells switching between growth and differentiation were studied.

Materials and Methods

Materials

NGF was purchased from Sigma Co. (St. Louis, MO). RPMI 1640 culture medium was purchased from Nissui Pharmaceutical Co., LTD. (Tokyo, Japan). Phospho-specific MAP kinase antibody (rabbit polyclonal IgG) was purchased from New England Biolaboratorys, Inc. (Beverly, MA). Vectastain avidin-biotinylated horseradish peroxidase complex (ABC) kit was purchased from Vector Laboratories Co. (Burlingame,CA). Diaminobenzidine (DAB) reagent set was purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Paraformaldehyde, Triton X-100 and sodium orthovanadate were purchased from Wako Pure Chemical Ltd. (Osaka, Japan). Horse serum and tris(hydroxymethyl) aminomethane (Tris) was purchased from GIBCO BRL Life Technology Co. (Osaka, Japan). Polyallylamine hydrochloride was purchased from Nittobo (Tokyo, Japan). Bovine serum albumin (BSA) was purchased from Intergen Co. (Purchase, NY). Anti-EGF IgG was purchased from Becton Dickinson Labware (Bedford, MA).

Preparation of Photo-Reactive Polyallylamine and Photo-Reactive EGF

The synthetic scheme of photo-reactive polyallylamine and photo-reactive EGF is shown in Figure 1. *N*-(4-azidobenzoyloxy)succinimide was prepared as described in chapter 2.

A phosphate-buffered solution (pH = 7.0, 10mL) containing polyallylamine (MW = 60,000, 30 mg) was added to the DMF solution (20 mL) of *N*-(4-azidobenzoyloxy)succinimide (25.8 mg) under stirring on ice. After reaction at 4 ℃ for 24 h under stirring, the solution was ultrafiltrated (Millipore MoleCut II, filtration off below 10 kDa) and washed once with 5 mL DMF/H_2O (2/1) solution and once with 5 mL distilled water. The azidophenyl-derivatized polyallylamine is referred to as AzPhPAAm. The content of azidophenyl group in the conjugate was calculated from the absorbance at 280nm.

The azidophenyl-derivatized polyallylamine was further conjugated with EGF as follows. To a 0.1 M 2-(*N*-morpholino)ethanesulfate-buffered solution (MES, pH=4.5, 10 mL), WSC (10 mg), EGF (300 μg) and AzPhPAAm (600 μg) were added, and reacted at 4 ℃ for 72 h under stirring. Then, the solution was ultrafiltrated (Millipore

MoleCut II, filtration off below 10 kDa) and washed twice with 2 mL distilled water. The photo-reactive EGF conjugate is referred to as AzPhPAAmEGF. The composition of EGF in the AzPhPAAmEGF conjugate was determined by measuring the fluorescence intensity at 345 nm by excitation at 280 nm.

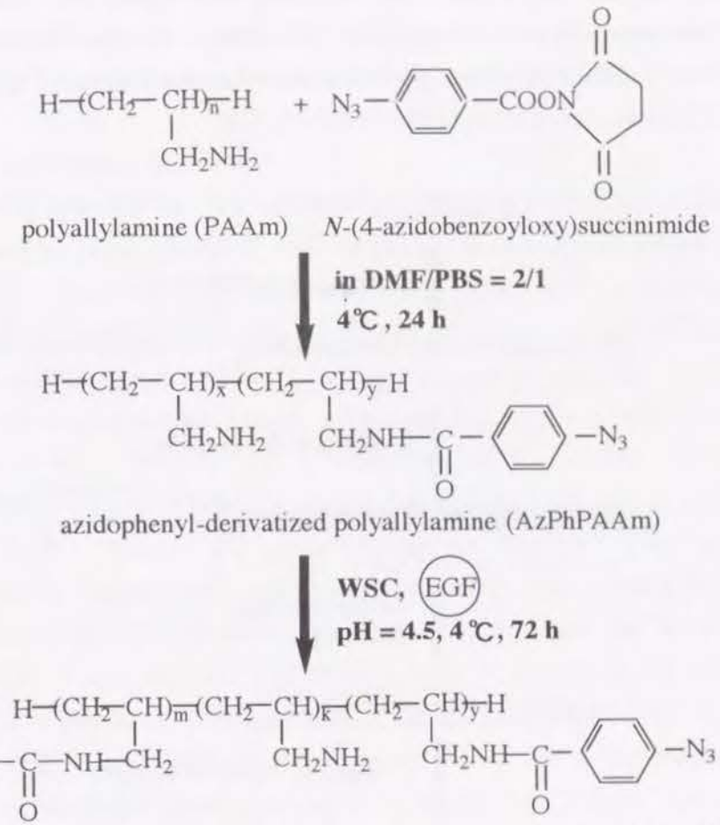

Figure 1 Synthesis of photo-reactive polyallylamine and photo-reactive EGF conjugate.

Immobilization of Fibronectin and EGF

Fibronectin (Fn) and EGF were co-immobilized on a polystyrene plate by the procedures as shown in Figure 2. An aqueous solution of AzPhPAAm (200 μg/mL) was placed in each of the six wells (diameter, 35 mm) of a PSt tissue culture plate. After air-drying, the plate was further coated with an aqueous solution of Fn (40 μg/mL, 100 μL) and air-dried. The plate was then irradiated for 10 s using a UV lamp (Koala 25, 100V) from a distance of 5 cm. Thereafter, the plate was repeatedly washed with PBS at 4℃ until the absence of AzPhPAAm and Fn release in the washing buffer was confirmed by

ultraviolet absorption at 280 nm (7 days). This Fn-immobilized PSt plate is referred to as Fn-PSt.

An aqueous solution of AzPhPAAm-EGF conjugate (200 μg/mL, 100 μL) was coated on each of the six wells of the Fn-PSt plate and air-dried at room temperature. Subsequently, the plate was irradiated for 10 s using the UV lamp from a distance of 5 cm. Thereafter, the plate was repeatedly washed with PBS at 4℃ until the absence of EGF conjugate release was confirmed by ultraviolet absorbance at 280 nm (7 days). The EGF-immobilized Fn-PSt plate is referred to as EGF-Fn-PSt.

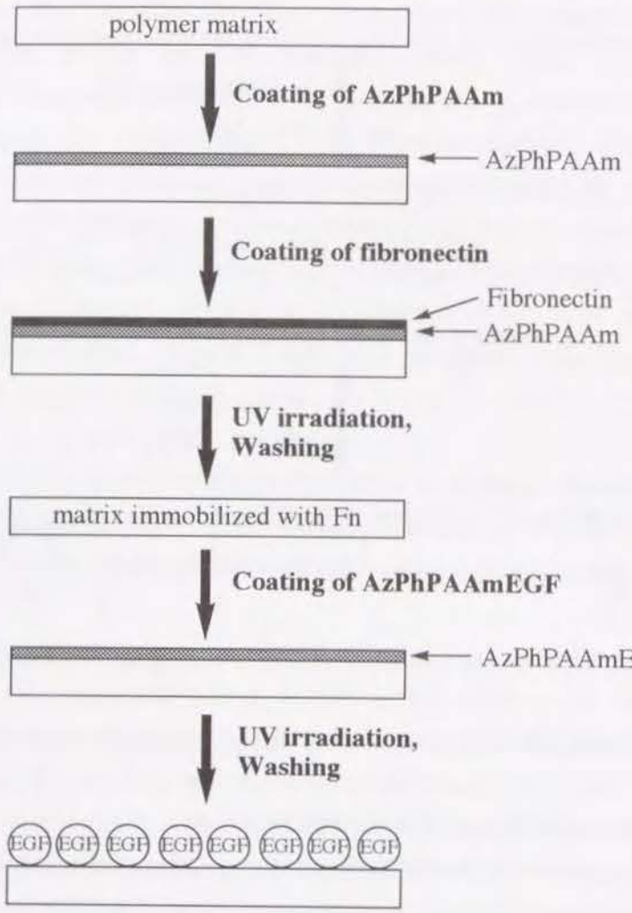

Figure 2 Schematic illustration of photo-immobilization of fibronectin and EGF.

Cell culture and Activation by NGF, EGF and Immobilized EGF

PC12 cells were subcultured in RPMI 1640 medium containing 5% (v/v)

FBS and 10% (v/v) horse serum under a 5% CO_2 atmosphere at 37 ℃. The subcultured PC12 cells from a single flask were harvested by pipetting. After washed twice with the medium mixture (DMEM medium/F12 medium = 1/1) containing transferrin (5 μg/mL), insulin (5 μg/mL) and progesterone (0.8 μg/mL), the cells were suspended in the medium (1×10^6 cells/mL). The cell suspension (0.1 mL/well) was added to each of six wells containing NGF (50 ng/mL, 5 mL), EGF (100 ng/mL, 5 mL) or immobilized EGF, respectively. The cells were cultured under a 5% CO_2 atmosphere at 37 ℃.

Assay of MAP Kinase Activity

PC12 cells that had been deprived of serum for 6 h were incubated at 37 ℃ for different periods of time in RPMI 1640 medium without serum in 6-well plates in the presence of NGF (50 ng/mL, 5mL), native EGF (100 ng/mL, 5mL) or immobilized EGF. After washing once with PBS, the cells were lysed in 500 μL of a solution containing 25 mM Tris-HCl (pH=7.4), 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 nM okadaic acid, 0.5 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF), and the lysate was centrifuged at 15,000 rpm for 15 min at 4 ℃. The MAP kinase activity of $p44^{mapk}$ was measured in immunoprecipitates prepared from cell lysate supernatants with polyclonal antibodies to MAP kinase (αC92)[13]. The immunoprecipitates were incubated for 10 min at room temperature with 1 μCi of [γ-^{32}P]ATP in a solution containing 25 mM Tris-HCl (pH=7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, 40 μM ATP, 0.5 mM EGTA, and myelin basic protein (MBP) (1 mg/ml) as a substrate[14]. Under these conditions, the MBP phosphorylation showed a linear time course for longer than 20 min. The reaction was terminated by adding 10 mL of stop solution containing 0.6% HCl, 1% BSA and 1 mM ATP, and 15 μL of reaction mixture was spotted onto P-81 phosphocellulose paper (Whatman), which was then washed three times with 0.75% phosphoric acid and once with acetone. The papers were dried, and ^{32}P was determined by Cerenkov counting.

Localization Assay of MAP Kinase

PC12 cells cultured for 60 min in the presence of NGF, native EGF or immobilized EGF were washed once with PBS and fixed by PBS containing 3% paraformaldehyde for 30 min at 4 ℃. The fixed cells were washed three times for 5 minutes each with 50 mM Tris-HCl buffered solution (pH 7.4) containing 150 mM NaCl, 0.1% Triton X-100 (TBST) and incubated with 1.5% normal goat serum in TBST (blocking buffer) for 60 min. Subsequently, the cells were incubated with phospho-

specific MAPK antibody (rabbit polyclonal IgG; 1/100 dilution in TBS containing 3% BSA) at 4 ℃ for 24 h, rinsed once for 5 min with 50 mM Tris-HCl buffer solution (pH 7.4) containing 150 mM NaCl (TBS), washed once for 15 min with TBST, once for 15 min with TBST containing 0.1% BSA and incubated with biotinylated anti-rabbit IgG (1 : 100 dilution in TBST containing 3% BSA) for 60 min at 25 ℃, washed three times for 5 min each with TBST, once for 5 min with TBS and incubated for exactly 30 min in 0.6% H_2O_2. After washing three times for 5 min each with TBST, the cells were incubated for 60 min with 1 mL ABC reagent at 25 ℃. The cells were washed three times for 5 min each with 1 mL of TBS, and then mixed with 1 mL of DAB reagent and the reaction progress was monitored under a microscope. Reaction was allowed to proceed for 10 min and terminated by adding 1 mL of water.

Results and Discussion

Activation of MAP Kinase

Time course of MAP kinase activation in PC12 cells is shown in Figure 3. Native NGF quickly activated MAP kinase and the effect continued for a time longer than 90 min after a little inactivation. Native EGF also quickly activated MAP kinase, but the activation was weakened soon. On the other hand, with immobilized EGF, the activation of MAP kinase took place a little slowly, but the activated state of the enzyme lasted longer than that with native EGF. The immoblilized EGF was even superior to NGF in activation of MAP kinase.

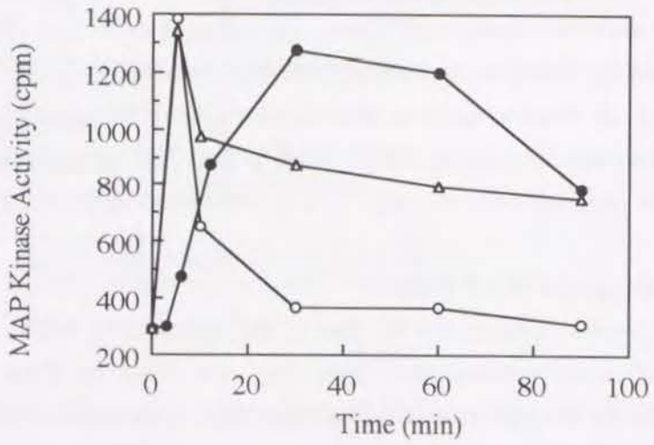

Figure 3 Time course of MAP kinase activation by native EGF (○), native NGF (△), and immobilized EGF (●).

Translocation of MAP Kinase

NGF promoted the translocation of MAP kinase from the cytosol to the nucleus of PC12 cells after 60 min incubation. In contrast, the translocation of MAP kinase to the nucleus was not significant in PC12 cells stimulated with native EGF. The extensive translocation of MAP kinase to the nucleus of PC12 cells by immobilized EGF is shown in Figure 4.

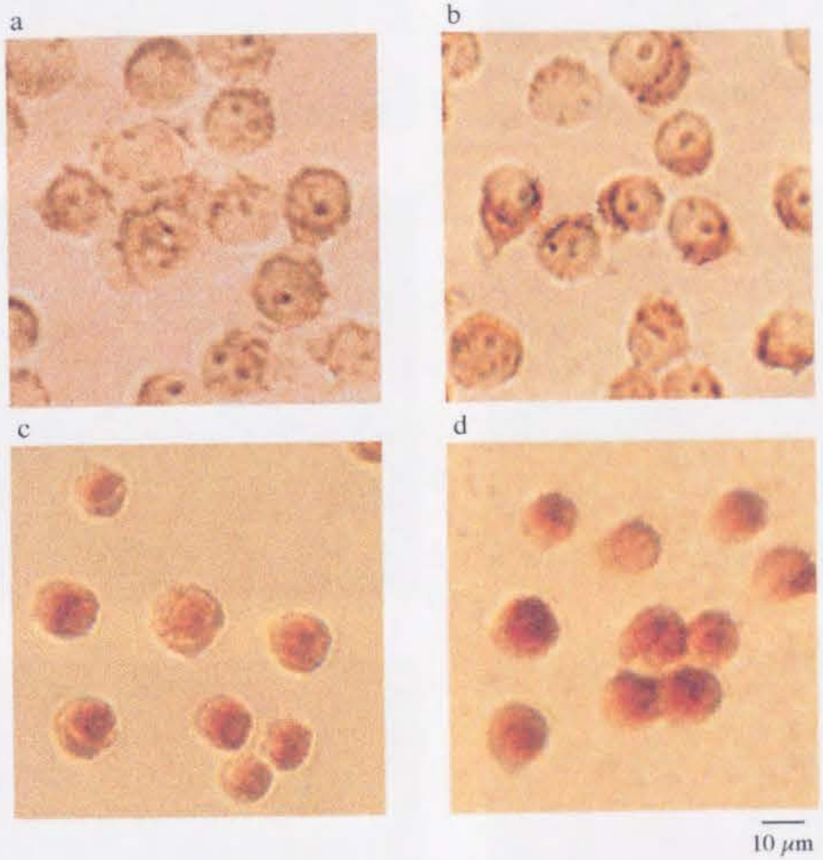

Figure 4 Translocation of MAP kinase from the cytoplasm to the nucleus of PC12 cells cultured (a) without any growth factor, or in the presence of (b) native EGF, (c) native NGF, and (d) immobilized EGF.

Growth and Differentiation

PC12 cells were cultured in the presence of native EGF, native NGF, or immobilized EGF. Phase-contrast micrographs of PC12 cells after 24-h and 72-h culture are shown in Figure 5. Native NGF and immobilized EGF induced neurite formation of PC12 cells, but native EGF did not.

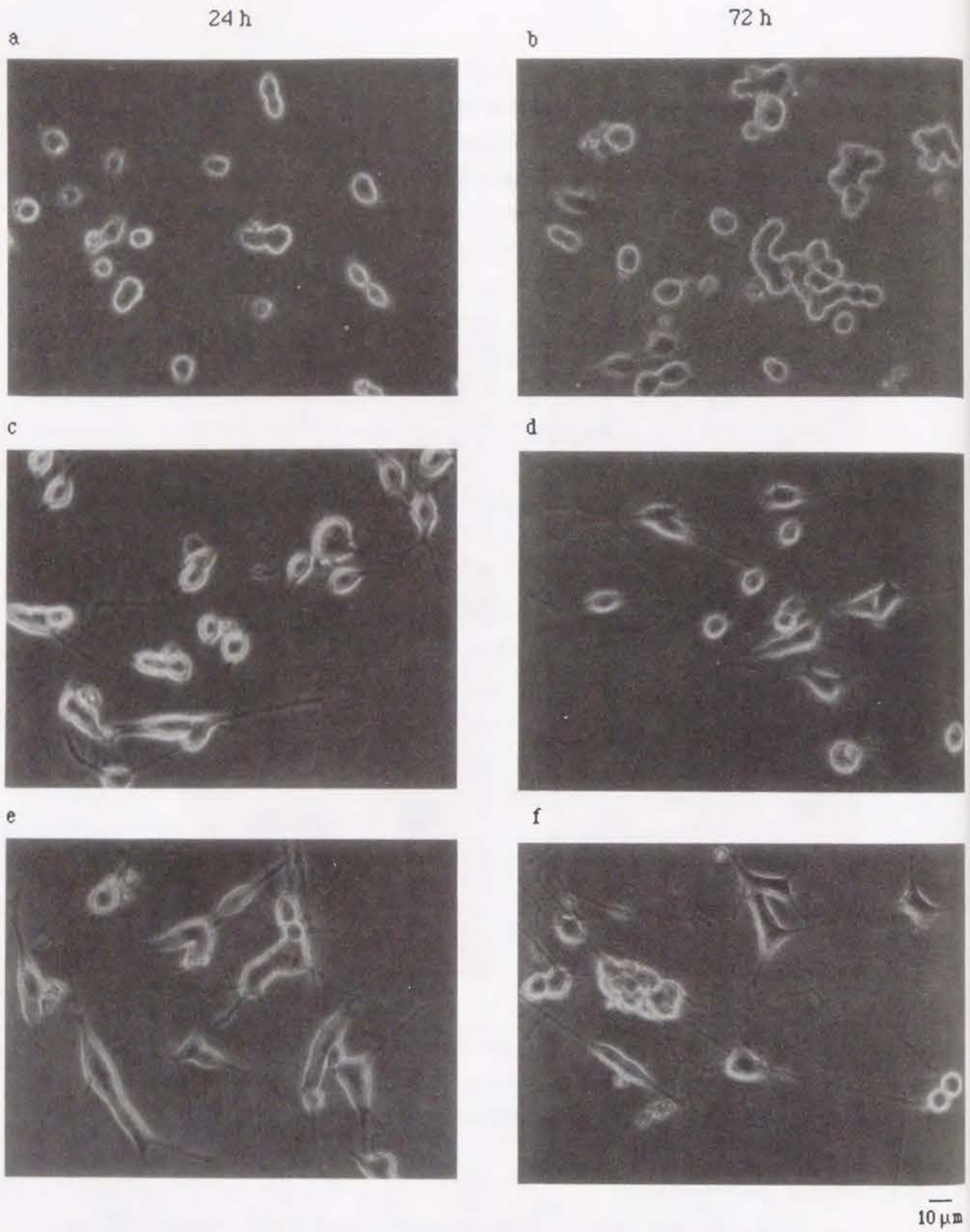

Figure 5 Phase-contrast micrographs of PC12 cells after culture for 24 h (a) and 72 h (b) in the presence of native EGF, 24 h (c) and 72 h (d) in the presence of immobilized EGF, and 24 h (e) and 72 h (f) in the presence of native NGF.

Table 1 summarizes the effect of native EGF, native NGF, and the immobilized EGF on the percentage of differentiation of PC12 cells. The immobilized EGF critically switched the cells from growth to neuronal differentiation. Traverse *et al.*[5] and Dikic *et al.*[6] have reported the neuronal differentiation of PC12 cells triggered by overexpression of EGF receptors and insulin receptors, respectively. They concluded that the overexpression induced long-lasting activation of MAP kinase, which is similar to the activation by NGF.

Table1 Effect of EGF and NGF on the neurite formation of PC12 cells

Sample	Neurite-bearing Cells (%)※	
	24 h	72 h
Native NGF	25±3	71±4
Native EGF	3±1	5±2
Immobilized EGF	26±2	59±5

※Neurite appearance was quantified by scoring 200 cells from randomly selected light microscope fields of the plates.

The present investigation also demonstrated that the activation of MAP kinase has some bearing on growth-differentiation switching. In addition, it was shown that the action of EGF, cell growth or cell differentiation, can be modulated by immobilization. Immobilization of EGF not only enhances cell growth of Chinese hamster ovary CHO-K1 cells and mouse fibroblast STO cells by suppression of internalization, but also turns the cell behavior of PC12 cells toward neuronal differentiation by realizing "juxtacrine activation".

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Part II

Thermal Control of Biomaterials Function: Cell Manipulation Using Thermo-Responsive Polymer

Chapter 5

Patterned Immobilization of Thermo-Responsive Polymer

Introduction

Micro-patterning of small molecules, macromolecules and cells on matrix surfaces has a wide range of potential applications in molecular electronics, micro-machining, biosensing, and tissue engineering[1-11]. To achieve the surface micro-patterning, various methodologies have been developed, which include photo-lithography[12-26], lithography using X-ray, electron beam, ion beam, and neutral metastable atom[27-29], mechanical stamping[30-34], electrochemical method[12], and the tip manipulation of scanning probe microscope[35-37].

On the other hand, signal-responsive polymeric networks have been interested in for use in micro-manipulators or articificial muscles[38-40]. So-called micro-machine might be fabricated by micro-processing of signal-responsive polymers. Poly(*N*-isopropylacrylamide) (PNIPAAm), a thermo-responsive polymer, has a low critical solution temperature (LCST) of about 32 ℃ in water[41]. PNIPAAm is fully hydrated with an extended chain conformation below 32 ℃ and is extensively dehydrated and compact over 32 ℃. Cross-linked PNIPAAm and its copolymers have been developed as thermal on-off switching polymers for drug permeation and release using this mechanism[42,43]. Okano *et al.*[44-46] and Takezawa *et al.*[47] have found that cell adhesion can be regulated on this thermo-responsive material by temperature. Cells are able to adhere onto hydrophobic surfaces, but do not adhere on highly hydrated hydrophilic surfaces.

In this chapter, PNIPAAm was immobilized in a specific pattern on a polystyrene plate and the regiospecific control of cell behaviors on the conjugate plate by temperature was investigated. Although regiospecific cell attachment to a matrix has been achieved[1,3,5,8], regiospecfic cell detachment has not been reported.

Materials and Methods

Materials

N-Isopropylacrylamide (NIPAAm) was kindly provided by Kohjin Co. (Tokyo, Japan). Mercaptopropionic acid (MPA) and 2,2'-azobisisobutyronitrile (AIBN) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). 4-Azidoaniline hydrochloride

was purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Photo-lithographic mask was purchased from Nippon Filcon Co., Ltd. (Osaka, Japan). NIPAAm and AIBN were recrystallized from hexane and methanol, respectively. MPA was purified by distillation *in vacuo*.

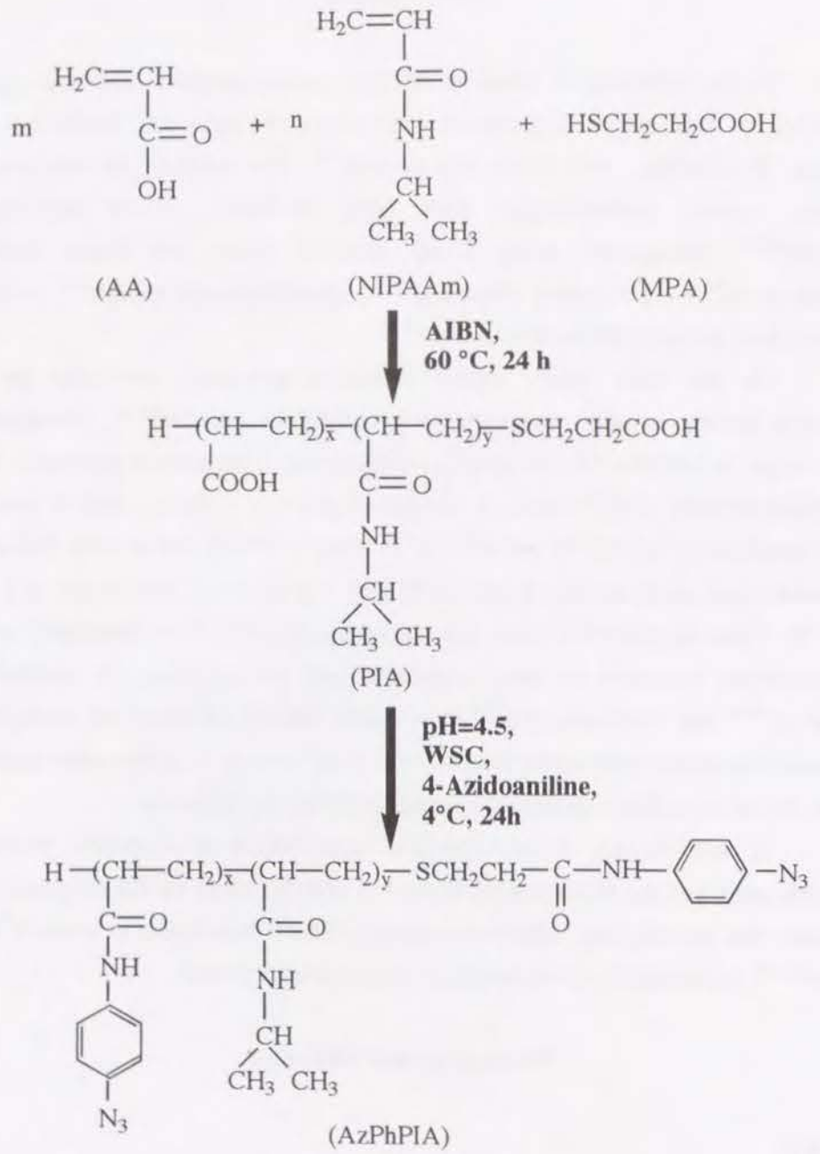

Figure 1 Synthetic scheme of azidoaniline-coupled thermo-responsive copolymer (AzPhPIA).

Copolymerization

The synthetic scheme of NIPAAm/AA copolymer and its azidophenyl derivative is shown in Figure 1. NIPAAm and AA were copolymerized using AIBN as an initiator in the presence of MPA as a chain-transfer reagent as described by Chen and Hoffman[48] and Takei *et al.*[49]. NIPAAm and AA at the molar ratio of 95/5 were dissolved in isobutyl alcohol (30 mL, total monomer concentration 2.0 M) containing AIBN (5 mg) and MPA (monomer/MPA = 100/4 mol/mol). The mixture was degassed, purged with nitrogen gas, sealed, and incubated at 60 ℃ for 24 h. The resulting mixture was diluted with methanol and the product copolymer was precipitated in diethyl ether. The NIPAAm/AA copolymer is referred to as PIA. The molecular weight was calculated from the sulfur content determined from titration with barium acetate after flask combustion, assuming that one terminal of a polymer chain is capped with MPA. Takei *et al.*[49] have reported that oligomers obtained by this method had an average one MPA residue per a chain of polymer according to gel permeation chromatography and analysis of the end group. The content of NIPAAm and AA in the copolymer was determined by elemental analysis.

The copolymer (50 mg), 4-azidoaniline (106.8 mg), and WSC (239.6 mg) were dissolved in 0.1 mM MES-buffered solution (5 mL, pH4.5) and the mixture was stirred at 4 ℃ for 24 h. Thereafter, the solution was centrifuged at 40 ℃. The precipitate was washed until the absence of azidoaniline in the washing solution was confirmed by ultraviolet spectroscopy. The azidophenyl derivative of PIA is referred to as AzPhPIA. The content of azidophenyl groups in the AzPhPIA copolymer was determined by ultraviolet spectroscopy (280 nm).

Micro-Processing

The micro-processing procedure is shown in Figure 2. A PSt plate (2 cm×2 cm) was cut from a tissue culture PSt flask (250 mL). An aqueous solution of AzPhPIA (10 mg/mL, 0.1 mL) was placed on the PSt plate and air-dried at room temperature. The plate was then irradiated with an ultraviolet lamp (Koala, 100 W) from a distance of 5 cm for 10 s through a patterned photomask or without a photomask. The plate was washed with cooled distilled water (10 ℃) until the absence of AzPhPIA release in the washing solution was confirmed by ultraviolet spectroscopy (280 nm).

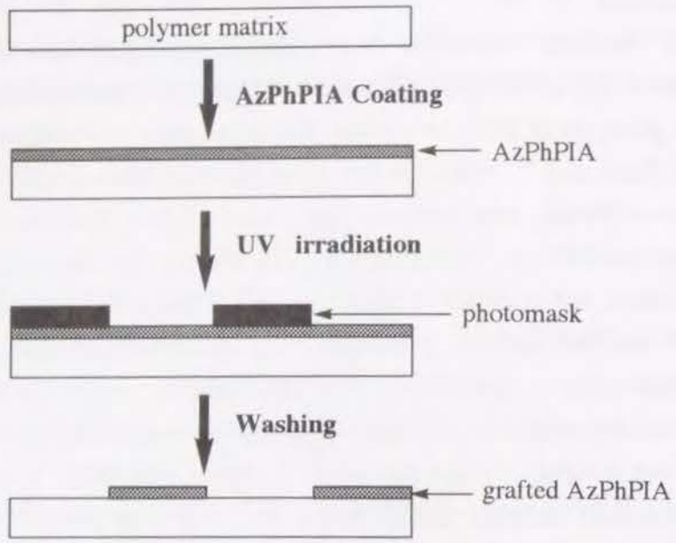

Figure 2 Procedure of photo-lithography using azidoaniline-coupled PIA copolymer.

Measurement of Temperature Sensitivity

The copolymer was dissolved in 0.1 M phosphate-buffered solution (pH = 7.0) to a concentration of 1.0 wt%. The turbility of the copolymer solution at various temperatures was monitored by the optical transmittance at 500 nm, which was recorded on a Hitachi spectrometer (Hitachi, Japan).

The contact angle of an air bubble placed on immobilized or untreated PSt plate in water was measured to assess surface wettability. The plate was incubated in distilled water at 10 ℃ or 37 ℃ for 24 h before the measurement.

The AzPhPIA-patterned PSt plate immersed in distilled water at 10 ℃ or 37 ℃ was observed using a phase-contrast microscope (Olympus Co., Tokyo, Japan).

Cell Culture

Subcultured mouse fibroblast STO cells were suspended in DMEM (1×10^6 cells/ml). The cell suspension (1.0 mL/well) was added to a 6-well culture plate containing AzPhPIA-micro-patterned PSt plates that had been incubated in the well in the presence of DMEM (5 mL) at 37 ℃ for 2 h. The cells were cultured under a 5% CO_2 atmosphere at 37 ℃ for 2 h. For changing temperature, the culture plate was kept at 10 ℃ for 30 min, keeping the medium unchanged. Subsequently, the medium was replaced with fresh medium. The cells before and after lowering temperature were observed by phase-contrast microscopy.

Results and Discussion

Synthesis of NIPAAm/AA Copolymer

The yield of NIPAAm/AA copolymer was 78%. The molecular weight was 5910 ± 30. The content of AA in the copolymer was the same as that in the feed (5 mol%). All of the carboxyl groups in the copolymer was coupled with azidoaniline. The derivatized copolymer contained about four azidophenyl groups in a chain in the present investigation.

On heating the aqueous solution of PIA, the transparency suddenly dropped. The aqueous PIA solution exhibited a LCST at 38.5 ℃ as shown in Figure 3a. The LCST

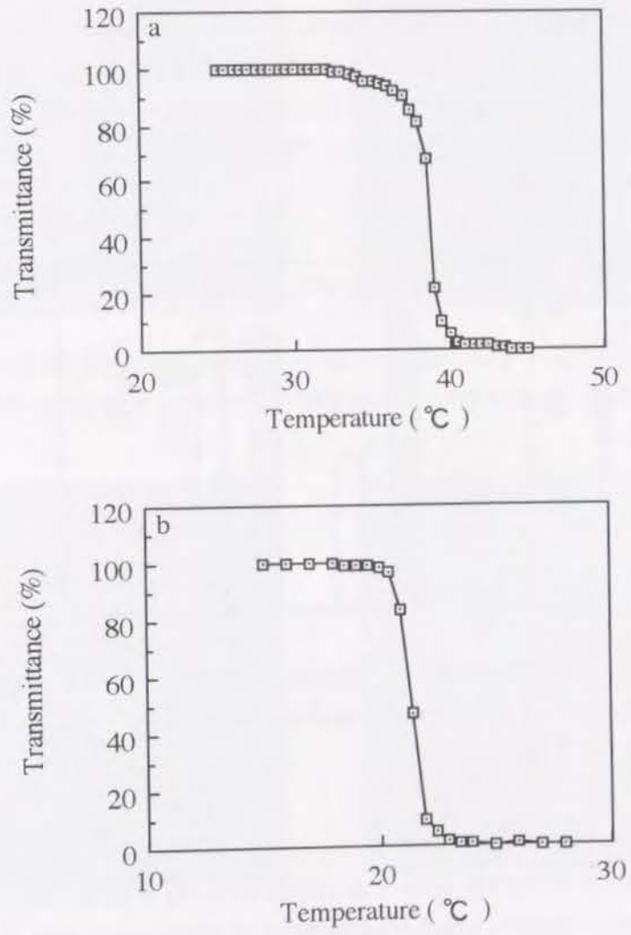

Figure 3 Temperature dependence of turbidity of an aqueous solution of (a) PIA copolymer and (b) azidophenyl-derivatized PIA copolymer.

of NIPAAm homopolymer has been reported to be 32 ℃[41]. The LCST increased with copolymerization of AA. Random copolymerization of NIPAAm with relatively hydrophilic monomers such as acrylamide and AA results in the rise of LCST, whereas the LCST of NIPAAm polymer is not significantly influenced by the molecular weight and solution concentration[50].

The LCST of AzPhPIA was 21.5 ℃ as shown in Figure 3b. Coupling with azidophenyl groups decreased the LCST by 17 ℃. The hydrophobilization of the copolymer should lower the LCST. It has been reported that random copolymerization of NIPAAm with hydrophobic monomers such as methyl methacrylate lowers the LCST[51].

Surface Property of AzPhPIA-Immobilized Plate

The wettability of the polystyrene plate immobilized with AzPhPIA depended on the temperature, while that of untreared polystyrene plate was not affected by temperature as shown in Figure 4. The wettability was related to the turbidity of the aqueous solution containing the copolymer. The graft chains should govern the thermal sensitivity of the plate surface.

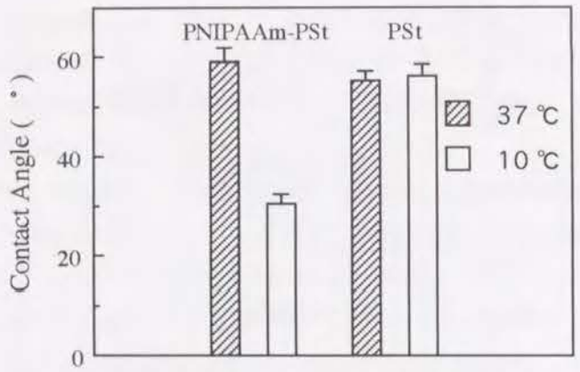

Figure 4 Water contact angle of a polystyrene plate (PSt) and an azidophenyl-derivatized PIA copolymer-immobilized polystyrene plate (PNIPAAm-PSt) at 10 ℃ and 37 ℃.

Micro-Patterned Immobilization

The micro-patterned plate was observed using a phase contrast microscope (Figure 5). The micro-pattern, which was the same as photo-mask, was observed at 37 ℃. Upon cooling the plate to 10 ℃, the regions of AzPhPIA graft escaped out of the sight by hydration of the graft chain.

Figure 5 Phase-contrast micrographs of the photomask used (a), and a PSt plate with azidophenyl-derivatized PIA immobilized in a specific pattern at (b) 37 ℃ and (c) 10 ℃.

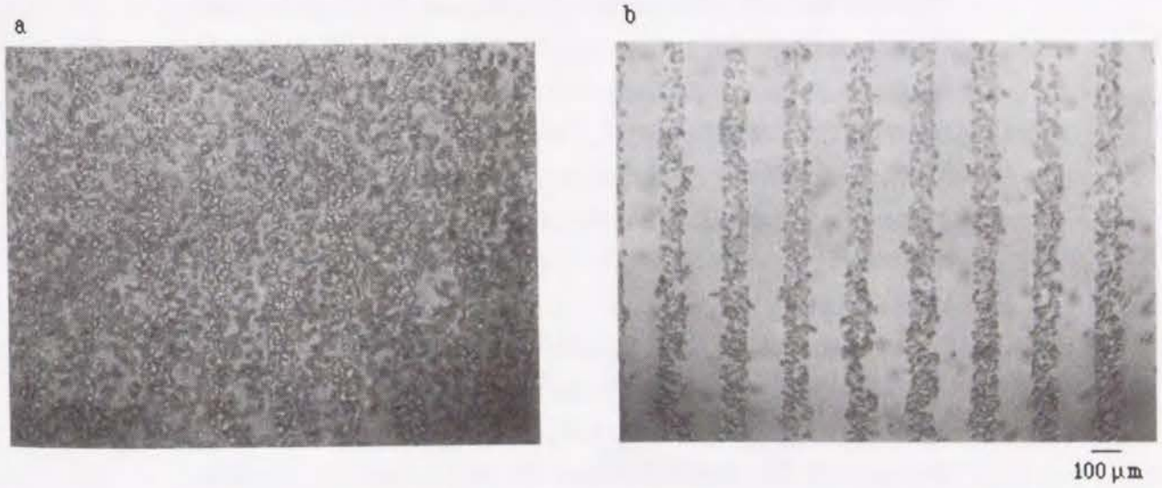

Figure 6 Phase-contrast micrographs of mouse fibrobalst STO cells cultured on the PSt plate with azidophenyl-derivatized PIA immobilized in a specific pattern at (a) 37 ℃ and (b) 10 ℃.

Selective Detachment of Cells from the Micro-Patterned Plate

STO cells were cultured on the micro-patterned plate until the cells reached constant adherence at 37 ℃ (Figure 6a). Subsequently, the culture system was cooled to 10 ℃. The cells adhered on the regions immobilized with AzPhPIA were selectively detached from the plate as shown in Figure 6b. At 10 ℃, regions immobilized with the AzPhPIA copolymer began to solvate and swell, changed to hydrophilic and the thermal on-off switch turned to the "detachment" mode, and adhesion of the STO cells to the regions was lost. However, the cells adhered on the regions without AzPhPIA immobilized kept unchanged during the manipulation of temperature, because of the absence of such a temperature-dependent hydrophobic-hydrophilic switch. Okano *et al.*[44-46] and Takezawa *et al.*[47] have reported that cell attachment and detachment on/from matrix grafted with thermo-responsive polymer can be regulated by temperature. The present investigation clearly demonstrated the selective detachment of cells from thermo-responsive polymer by micro-patterning.

In this investigation, the micro-patterning of signal-responsive polymer was used for tissue engineering. The combination of micro-patterning technic with various types of polymers will be useful for development of micromachines.

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Chapter 6

Cell Growth and Adhesion Regulated by Insulin Conjugate with Thermo-Responsive Polymer

Introduction

The culture of mammalian cells is a key technique for both basic research in biology and industrial applications. The importance of substrata or biomaterials, such as biological and synthetic extracellular matrices for cell culture as well as for the production of artificial tissues and organs has been increasing. However, it is difficult to regulate cellular functions, such as proliferation, secretion, movement, and differentiation with biomaterials alone. The immobilization of biosignal molecules such as growth factors and cytokines in such a manner that they retain their activity would therefore significantly contribute to the design of biomaterials.

It was shown that insulin immobilized on various artificial and biological substrata increases the growth of anchorage-dependent cells (mouse fibroblast, bovine endothelial, mouse sarcoma, and Chinese hamster ovary cells) to a greater extent than native insulin[1-9]. These findings demonstrate that cells can be cultured on artificial substrata without including serum in the culture medium.

On the other hand, most mammalian cells must adhere to a solid substratum or supporting scaffold in order to proliferate and manifest their functions[10-12]. In these *in vitro* cell culture systems, efficient recovery of cells from the culturing substrata is an essential process for their further utility. Generally, recovery of cultured cells from substratum requires treatment with a proteolytic enzyme such as trypsin. The enzymatic treatment inflicts damage to cell membranes by hydrolyzing various membrane-associated proteins, resulting in impairment of cell function[13-15]. Therefore, cultured cells sensitive to enzymatic treatment can not be subcultured and only primary cells are utilized for various research purposes[16]. By patterned immobilization of thermal-responsive poly(*N*-isopropylacrylamide) on a PSt plate, the regiospecific cell detachment was obtained without using any enzymes as described in chapter 5.

In this chapter, the two design characteristics of biomaterials described above were combined by coupling insulin with thermo-responsive polymer on a PSt matrix. The composite material may not only enhance cell growth in the absence of serum but also allow simple recovery of the viable cells by manipulating the temperature.

Materials and Methods

Copolymerization and Insulin Coupling

The synthetic scheme of the insulin-conjugated NIPAAm/AA copolymers (Ins-PIA) is shown in Figure 1. NIPAAm and AA at various molar ratios were copolymerized and the molecular weight of the synthesized copolymers was determined as described in chapter 5. The molar ratio of NIPAAm and AA in the copolymers was determined by elemental analysis.

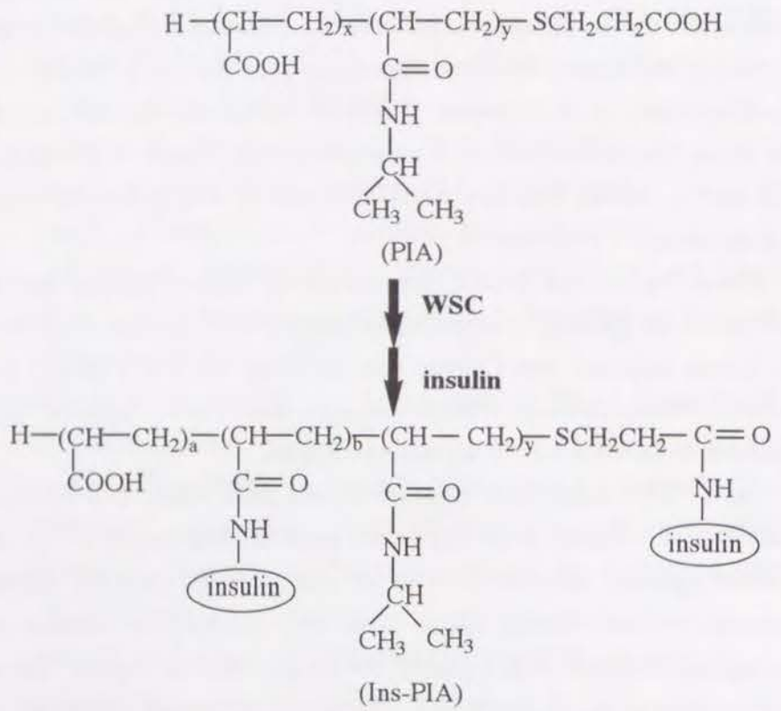

Figure 1 Synthetic scheme of insulin-conjugated thermo-responsive PIA copolymers.

One milliliter of an aqueous solution of the PIA copolymers (100 μg) was mixed with WSC (1 mg) in borate-buffered solution (1 mL, pH 7.0) and stirred at 4 ℃ for 24 h. The activated polymer was rapidly purified by ultrafiltration (Millipore MoleCut II, filtration off less than 3 kDa), and mixed with an aqueous solution of insulin (1 mg/mL) with or without ^{125}I-insulin, then stirred at 4 ℃ for 24 h. Finally, the activated carboxylic acid groups of PIA copolymers that remained unreacted were blocked by

mixing with glycine (10 mg/mL). The products were purified by ultrafiltration. The insulin-conjugated PIA copolymers were referred to as Ins-PIA and analyzed by HPLC [column packed with Cosmosil 5Diol-120 (Nacalai Tesque Inc., Kyoto, Japan); eluent, 0.02 M phosphate buffer (pH 7.0) containing 0.2 M NaCl; elution rate, 1.0 mL/min; temperature, 20 ℃; detection, absorbance at 280 nm].

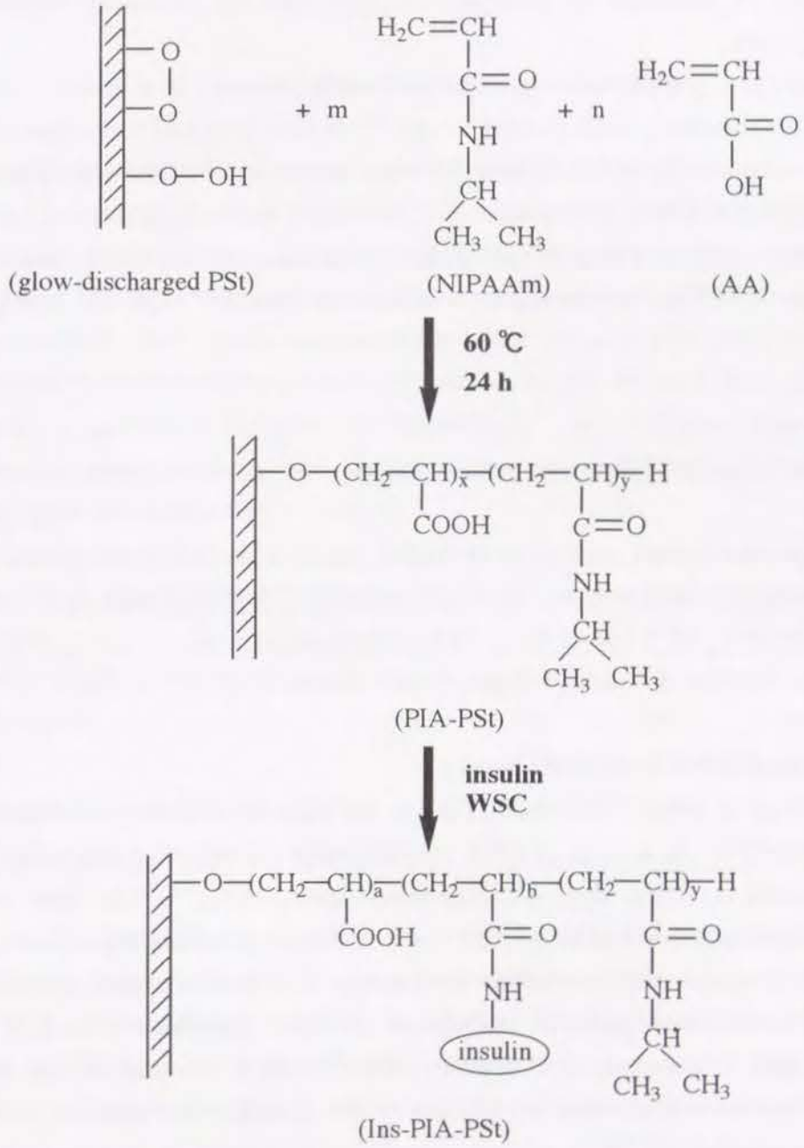

Figure 2 Synthetic scheme of insulin-NIPAAm/AA copolymer-grafted PSt film.

Graft Polymerization and Insulin Immobilization

The synthetic scheme of Ins-PIA-immobilized polystyrene film is shown in Figure 2. A PSt film was cast from toluene solution (15 wt%) on a circular glass plate (diameter, 15 mm). The film was glow-discharged and incubated in an isobutyl alcohol solution (30 mL) of NIPAAm and AA at various molar ratios (total monomer concentration, 2.0 M) at 60 ℃ for 24 h. The grafted film was washed with distilled water until no release of monomer or polymer was confirmed by measuring ultraviolet absorbance (220 nm).

The films grafted with PIA copolymer were immersed in a borate-buffered solution (pH 7.0) containing WSC (1 mg/mL) and ^{125}I-insulin at various concentrations at 4 ℃ for 48 h under stirring. After the immobilization procedure, the film was repeatedly washed with PBS (pH 7.4) until the release of ^{125}I-insulin in the washing solution ceased. Non-radiolabeled insulin was immobilized under the same conditions. These films were disinfected with 70% (v/v) ethanol, and washed with sterilized PBS for biological experiments.

Measurements

The turbility of the copolymer solutions was monitored as described in chapter 5.

The water contact angle of an air bubble placed on grafted or non-grafted PSt films in water was measured to assess the surface wettability. The films were incubated in distilled water at 10 ℃, 37 ℃, or 45 ℃ for 24 h before measurement.

The ESCA was measured as described in chapter 1.

Cell Culture and DNA Synthesis Assay

Culture of Mouse fibroblast STO was the same as described in chapter 1. After culture for 48 h, the amount of DNA synthesized in the cells was determined by complex formation with the dye, 4',6-diamidino-2-phenylindole[8,9]. The cells were solubilized by heating in 1 M NaOH (0.5 mL) at 70 ℃ for 30 min or until complete lysis was confirmed by means of phase-contrast microscopy. The lysate was then neutralized with 1 M HCl and homogenized. The homogenate (100 μL) was added to 3 mL of 10 mM Tris-HCl (pH 7.0) containing 100 mM NaCl, 10 mM EDTA and the dye (100 ng/mL). The fluorescence intensity at 450 nm of the complex was measured at an excitation wavelength of 360 nm.

Cell Detachment from the Matrix

After culture for 48 h, the cells were incubated for 30 min at 10 or 20 ℃ and for a further 5 min at 20 ℃ in the same medium. The cells before lowering temeperature (viable cells) and the detached cells were collected and the amount of DNA in the two cases was determined using the dye as described above. The cell detachment (%) was calculated by dividing the amount of DNA in detached cells by the amount of DNA in the viable cells.

Results and Discussion

Synthesis of Insulin-Conjugated NIPAAm/AA Copolymers

Figure 3 shows the HPLC elution pattern of insulin and its conjugates with thermo-responsive PIA copolymers, which was monitored by the insulin absorption at 280 nm. The molecular weight estimated from the position of the eluted peak indicated that one molecule of insulin was coupled to one molecule of each PIA copolymer. Insulin contains three free amino groups, referred to as A1, B1 and B29. Considering the reactivity reported by Schuttler and Brandenburg[17], the copolymer probably coupled to the most reactive A1-glycine. The properties of synthesized copolymers and their conjugates are summarized in Table 1.

Table1 Properties of NIPAAm/AA copolymers and the insulin conjugates

Sample	PIA-0	PIA-1	PIA-5
NIPAAm/AA(mol/mol) in the feed	100 : 0	99 : 1	95 : 5
Yield (wt%)	74	80	78
Mn	3790±30	6090±30	5910±30
Content of AA(mol/mol copolymer)	0.0	1.0	3.0
Coupled insulin (mol/mol copolymer)	1.0	1.0	1.0

On heating aqueous solutions of NIPAAm/AA copolymers, the transparency suddenly dropped. They exhibited a lower critical solution temperature (LCST). The LCSTs of PIA-0, PIA-1, and PIA-5 were 31.6 ℃, 33.5 ℃, and 38.5 ℃, respectively. The LCST increased with increasing the AA content in the copolymers. Random copolymerization of NIPAAm with relatively hydrophilic monomers such as acrylamide and AA results in the rise of LCST, whereas the LCST of NIPAAm polymer is not significantly influenced by the molecular weight and solution concentration[18].

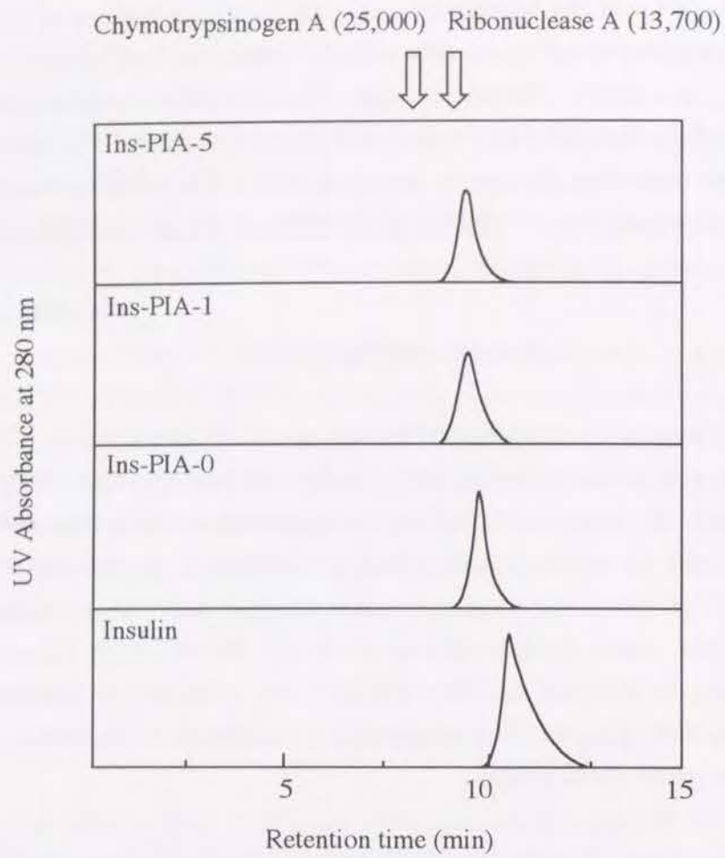

Figure 3 HPLC elution profiles of insulin and its conjugates. The notations are given in Table 1. The arrows indicate the retention times of reference proteins. The numbers in parentheses indicate the molecular weight.

The LCSTs of insulin-conjugated PIA-0, PIA-1, and PIA-5 copolymers were 32.2, 34.2, and 39.2 ℃, respectively. Coupling with insulin increased the LCST by the range of 0.6 - 0.7 ℃. The content of AA in the copolymer markedly affected the LCST, whereas insulin coupling did not. Chen and Hoffman[19] have reported that the random copolymerization of NIPAAm and AA significantly increased the LCST of the copolymer, whereas block copolymerization did not. Since insulin coupling is considered as a kind of block copolymerization, the nature of insulin may not affect the LCST as a block segment. Similar results have been reported by Hoffman and coworkers[20,21] and Matsukata *et al.*[22], who synthesized enzyme conjugates with NIPAAm copolymers. Stayton *et al.*[23] recently reported that the streptavidin (15 kDa) coupled with NIPAAm polymer (3.8 kDa) exhibited a LCST.

Graft Copolymerization and Insulin Immobilization

The ESCA of polystyrene films with various surface modifications was analyzed, and the results are shown in Table 2. The content of oxygen in the film surface increased after the glow discharge procedure. After graft copolymerization with NIPAAm and AA, nitrogen was detectable. The composition of the graft copolymers did not significantly influence the results of the surface analysis by ESCA. Insulin immobilization increased the content of both oxygen and nitrogen.

Table 2 ESCA measurement of surface-treated PSt films

Sample	NIPAAm : AA in the feed (mol : mol)	Element Composition (%) C	O	N
PSt		97.8	2.2	0.0
Glow-discharged	-	83.7	16.3	0.0
PIA-0-Pst	100 : 0	77.5	11.0	11.5
PIA-1-Pst	99 : 1	77.9	11.3	10.8
PIA-5-Pst	95 : 5	78.3	11.4	10.3
Ins-PIA-1-PSt※	99 : 1	65.4	18.5	16.1

※PIA-1-PSt immobilized with insulin (0.318 $\mu g/cm^2$)

The amount of insulin immobilized onto the surface-grafted polystyrene film is shown in Figure 4. With increasing insulin concentrations in the feed mixture, the amount of immobilized insulin also increased. The maximal surface concentration of insulin was about 0.4 $\mu g/cm^2$. Claesson *et al.*[24] have reported that insulin adsorbed on surfaces forms a monolayer at a concentration of 0.18 - 0.34 $\mu g / cm^2$.

The wettability of the PSt film modified with PIA depended on the temperature, while that of glow-discharged PSt film was not affected by temperature as shown in figure 5. When the temperature changed from 10 ℃ to 37 ℃, the surface of the PIA-1-PSt and Ins-PIA-1-PSt films having graft chains of a lower LCST changed from hydrophilic to hydrophobic, whereas the surfaces of the PIA-5-PSt and Ins-PIA-5-PSt films having graft chains of a higher LCST remained hydrophilic and became relatively hydrophobic when the temperature was raised to 45 ℃. The temperature dependence of wettability was closely related to the LCST of the grafted copolymer chains. The graft chains should govern the thermal sensitivity of the grafted film surface. However, the wettability should be altered by the amount of grafted copolymer, although the LCST of the copolymer solution is not significantly affected by the concentration.

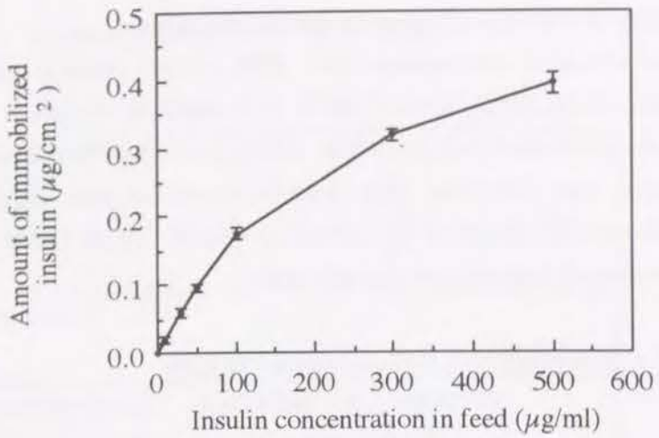

Figure 4 Amount of insulin immobilized onto PIA-1-PSt film. The amount was determined from the radioactivity level of ^{125}I-insulin on PIA-1-PSt film. Bars represent the standard deviations. n = 5.

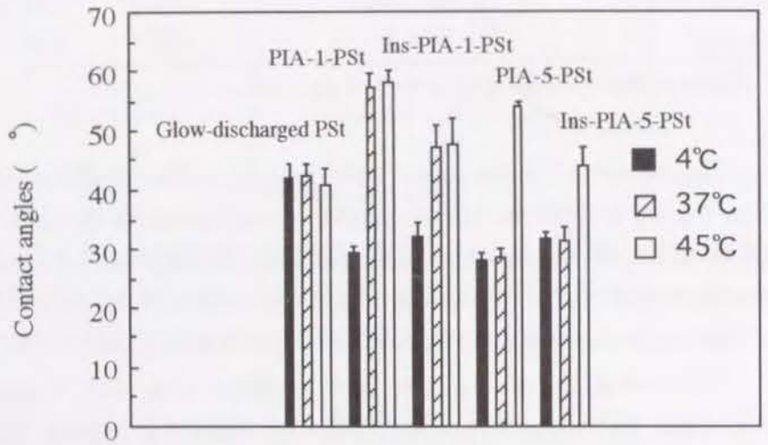

Figure 5 Water contact angles of PSt film surfaces at several temperatures. The increase of contact angle indicates the decrease of surface wettability. The notations are given in Table 2. The bars represent the standard deviations. n = 15.

Insulin immobilization did not significantly affect the thermal sensitivity of the film. Considering that the amount of immoblized insulin was higher than the order of theoretical insulin monolayer as described above, the copolymer should extend out to the surface between gap spaces formed by immobilized, rigid insulin.

Cell Culture on Modified Films

Far less immobilized insulin than native insulin was required to attain the same extent of acceleration of DNA synthesis in STO cells, as shown in Figure 6. In addition, the immobilized insulin was a little more mitogenic than the native form. No significant release of immobilized insulin from the films was detected using ^{125}I-insulin. Ito *et al.*[4] have reported that the insulin immobilized onto a surface-hydrolyzed poly(methyl methacrylate) film stimulates the insulin receptor and the related signal transduction proteins for a longer period than native insulin by inhibiting down-regulation of the insulin receptors caused by internalization. The immobilized insulin in this study should also stimulate the systems for a longer period accounting for the high mitogenic activity.

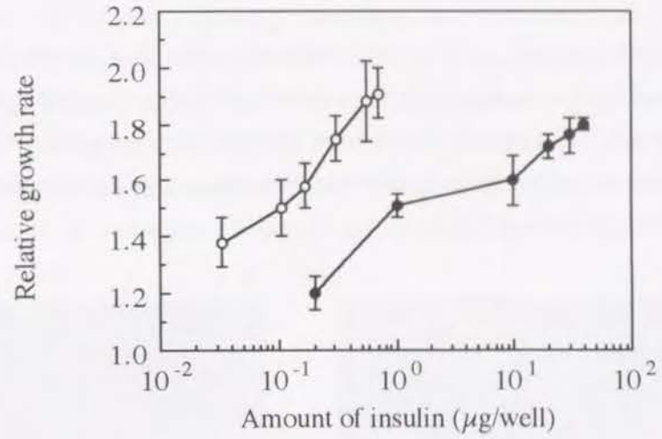

Figure 6 DNA synthesis in mouse fibroblast STO cells cultured in DMEM for 48 h on PIA-1-PSt in the presence of native insulin (●) and on Ins-PIA-1-PSt (○). The amount of DNA synthesized in the cells cultured on PIA-1-PSt in the absence of insulin was taken 1.0. Bars represent standard deviations. n = 8.

The cell-culture system was cooled to 10 or 20 ℃ for 30 min. Cells did not detach from glow-discharged polystyrene, PIA-5-PSt or Ins-PIA-5-PSt films, the surface properties of which were not influenced by cooling from 37 ℃ to 10 or 20 ℃ (Table 3). However, about fifty percent of the vaible cells detached from PIA-1-PSt or Ins-PIA-1-PSt films, the surfaces of which switched from hydrophobic to hydrophilic accompanying the temperature change from 37 ℃ to 10 ℃ or 20 ℃. No significant effect of cooling (10 or 20 ℃) was observed.

Table 3 Cell detachment (%)[1] from modified films by incubation at 10 or 20 ℃ for 30min

Sample[2]	Low-temperature treatment	
	10 ℃	20 ℃
Glow-discharged PSt	1.4±1.0	1.6±1.3
PIA-1-Pst	53.0±10.4	55.3±4.7
Ins-PIA-1-Pst	42.2±1.0	48.1±5.1
PIA-5-PSt	2.9±0.9	1.8±1.1
Ins-PIA-5-PSt[3]	0.0	2.3±0.9

1. Data are means ± SD, n = 8.

2. Notations are given in Table 2.

3. PIA-5-Pst immobilized with insulin (0.340 μg / cm^2).

The morphology of STO cells cultured on Ins-PIA-1- PSt film is shown in Figure 7. The cells at low temperature were round and easily detached from the film. The cytoskeleton of the cells should have been re-organized by the perturbation of cell membrane. However, the detached cells were transferred to a new culture medium, in which they proliferated normally.

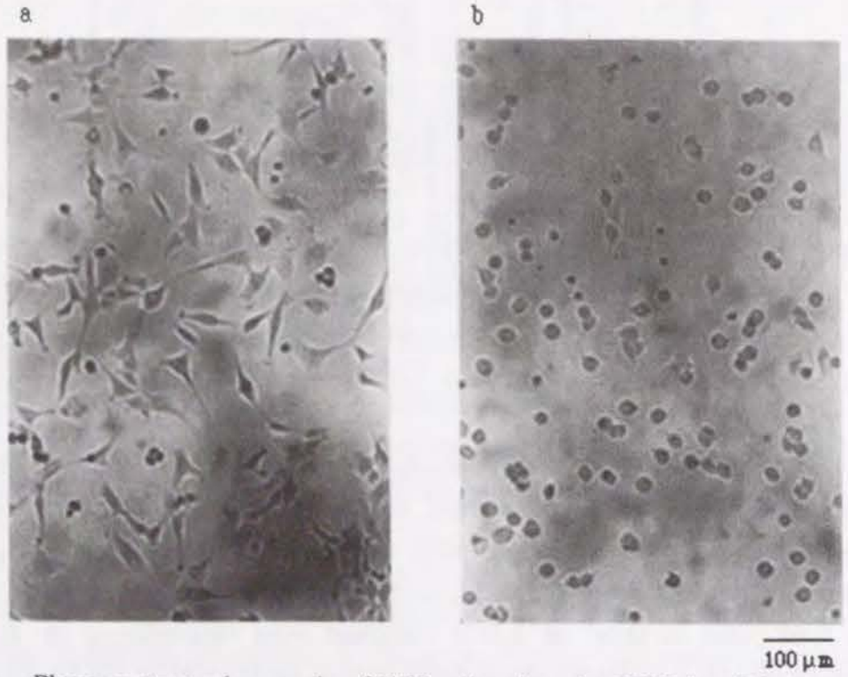

Figure 7 Phase-contrast micrographs of STO cells cultured at 37 ℃ for 48 h (a), and then incubated at 10 ℃ for 30 min (b) on Ins-PIA-1-PSt.

Vuori and Rouslahti[25] have demonstrated that integrin specifically binds to insulin receptor substrate-1 that binds the signaling molecules Grb2 and phosphatidylinositol-3' kinase in cells stimulated by insulin and vitronectin. This provided evidence of synergy between integrin and growth factor pathways. Ito *et al*[8,9] also found that cell growth is significantly enhanced by co-immobilization of growth factor and adhesion factor. However, cell adhesion and spreading are not significantly affected by the immobilized insulin[2]. This result indicates that the signal from immobilized insulin did not induce the re-organization of cytoskeleton proteins leading to adhesion and spreading. In addition, the interaction between insulin and the insulin receptor is considered to be weaker and less than that among cytoskeletons organized through the adhesion process. Therefore, we considered that the perturbation of cytoskeleton structure by thermo-responsive polymers overruled the insulin-insulin receptor interaction and induced dissociation of the complex. Takezawa and Yoshizato[26] have reported that hepatocytes adhered to a substrate mixed with NIPAAm polymer and collagen, and that viable cells were easily recovered by lowering the temperature regardless of the presence of collagen. This result indicates that the perturbation induced by the thermo-responsive polymer can reduce even the interaction between the extracellular matrix and integrin. Thermo-responsive bioconjugated materials will provide a new system for cell-culture engineering.

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Part III

Geographical Control of Biomaterials Function: Micro-Patterning of Cell Proliferation

Chapter 7

Artificial Juxtacrine Stimulation on Cell Growth by Patterned Immobilization of Insulin

Introduction

Natural and artificial substrata are important in basic bioscience and biotechnology including cell culture and tissue engineering[1-3]. Cellular interactions with the extracellular matrix play critical roles in various biological processes, including migration, morphogenesis, growth, differentiation and apoptosis[4]. On the other hand, selective cell attachment[5-8], selective cell detachment and cell shape regulation[9] by micro-patterned surface substrata have been reported. However, those artificial substrata could not transduce biological signals such as for growth and differentiation. It is shown in chapters 1 and 2 that immobilized insulin enhanced growth of anchorage-dependent cells to a greater extent than soluble protein. The immobilized insulin activated the insulin receptor and downstream signaling proteins, and this activation persisted for a longer period than that obtained with native insulin, probably explaining the greater mitogenic effect of the immobilized insulin[10]. In this chapter, the signal transduction from the immobilized insulin was clearly visualized and the possibility to regulate cell functions through "artificial juxtacrine stimulation" was examined using insulin immobilized in a prescribed micro-pattern.

Materials and Methods

Materials

Poly(acrylic acid) (PAA, MW = 450,000) was purchased from Aldrich Chem. Co. (Milwaukee., WI). Poly(ethylene terephthalate) film was purchased from Akita Sumitomo Bake Co. (Akita, Japan). Anti-insulin IgG was purchased from Kyowa Medics (Tokyo, Japan). Anti-phosphotyrosine antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rhodamine conjugated antibody was purchased from Protos Immunoresearch (San Francisco, CA). Vectashield™ mounting medium for fluorescence was purchased from Vector Laboratories, Inc. (Burlingame, CA).

Preparation of Photo-Reactive Poly(acrylic acid) (AzPhPAA).

PAA (1.0 mmol of monomer unit), 4-azidoaniline (0.2 mmol), and WSC (6.0 mmol) were dissolved in deionized water (110 mL) and stirred at 4 ℃ for 48 h. After the reaction mixture was concentrated under reduced pressure, dialysis was conducted using a seamless cellulose tube (cut off below 12 kDa). The dialyzed polymer was freeze-dried *in vacuo* to obatin 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 around 7 ppm and those of the methylene protons of the AzPhPAA main chain at 1.3 ppm.

Microprocessing and Immobilization of Insulin.

Insulin was immobilized on a poly(ethylene terephthalate) film in a prescribed pattern as shown in Figure 1. A water/methanol (2/3, v/v) mixed solution containing AzPhPAA (5 mg/mL) was cast on a poly(ethylene terephthalate) film (diameter, 13.5 mm) and air-dried. The film was covered with a photomask in a prescribed pattern and was UV-irradiated for 10 s using a UV lamp (Koala, 100 W) from a distance of 5 cm. The film was thoroughly washed with PBS. And then, the film was immersed in a MES-buffered solution (pH=4.5, 0.1 M, 10 mL) containing WSC (1 mg/mL) at 4 ℃ for 6 h. After washing, the film was incubated in insulin solution (1 mg/mL, 10 mL) at 4 ℃ for 24 h. The immobilized insulin film was washed with PBS until absence of insulin release in the washing solution was confirmed by ultraviolet spectroscopy at 280 nm.

Observation of Pattern of Insulin by Immunofluorescence Micoscopy

The film immobilized with insulin in a prescribed pattern was immersed in PBS containing 0.02% NaN_3 and 3% BSA at 4 ℃ for 24 h. Then the film was incubated in PBS containing anti-insulin mouse IgG antibody (2 μg/mL), 0.02% NaN_3 and 3% BSA at 4 ℃ for 12 h. After washing with PBS containing 0.02% NaN_3, the film was incubated in PBS containing rhodamine-conjugated anti-mouse IgG antibody (2 μg/mL) and 0.02% NaN_3 at 4 ℃ for 12 h. The stained film was washed with PBS and was observed under a fluorescence microscope (Olympus Co., Tokyo, Japan).

Cell Culture

Chinese hamster ovary cells overexpressing insulin receptors (CHO-T, 10^6 receptors per cell) were subcultured in Ham's F-12 medium containing 10% (v/v) FBS under a 5% CO_2 atmosphere at 37 ℃. The subcultured CHO-T cells were harvested by incubation at 37 ℃ for 5 min with PBS containing 0.02% (w/v) EDTA and 0.15% trypsin.

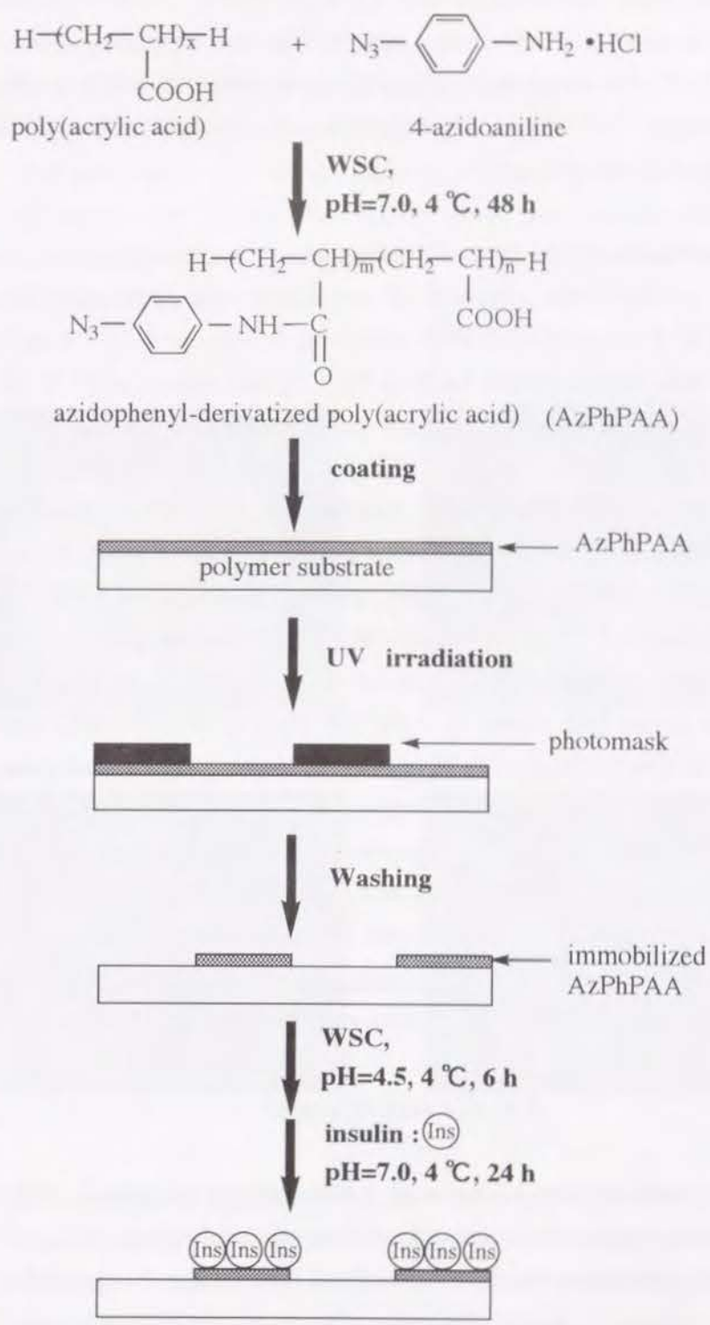

Figure 1 Procedure of patterned immobilization of insulin.

After washing once with Ham's F-12 medium containing FBS and once with Ham's F-12 medium, the cells were suspended in Ham's F-12 medium (1×10^5 cells/mL). The cell suspension (1 mL/well) was added to a sample film and incubated under a 5% CO_2 atmosphere at 37 ℃ for three days. The cells were observed under a phase-contrast microscope (Olympus Co., Tokyo, Japan) before and after culture. The cell density was counted under the microscope.

Observation of Cells by Immunofluorescence Microscopy

The CHO-T cells deprived of serum for two days were harvested by incubation at 37 ℃ for 10 min with PBS containing 0.02% (w/v) EDTA and cultured in the presence of immobilized insulin under a 5% CO_2 atmosphere at 37 ℃ for 8 h. The film adhered with cells was incubated in 3% paraformaldehyde for 30 min at 4 ℃. The fixed cells were washed three times with PBS containing 1 mM Na_3VO_4. Subsequently, the cells were permeabilized with PBS containing 0.25% Triton X-100 and 1 mM Na_3VO_4, and washed three times with 50 mM Tris-HCl-buffered solution containing 150 mM NaCl and 0.1% Triton X-100 (TBST, pH = 7.4) and 1 mM Na_3VO_4. After an overnight incubation at 4 ℃ in TBST containing 1.5% normal goat serum and 1 mM Na_3VO_4, the treated cells were incubated for 2 h at 25 ℃ with a solution of anti-phosphotyrosine mouse IgG diluted to 1/100 fold with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.01% Tween 20, 0.02% NaN_3, 1 mM Na_3VO_4 (TBS) containing 3% BSA. The cells were washed once with TBS, once with TBST and once with TBST containing 0.1% BSA. A solution of rhodamine-conjugated anti-mouse IgG antibody was diluted to 1/200 fold with TBS containing 3% BSA and incubated with the cells at 25 ℃ for 2 h. The cells were washed three times for 5 min each with TBST, three times with PBS, briefly rinsed with distilled water, and mounted in Vectashield mounting medium. The cells were observed by a fluorescence microscope (Olympus Co., Tokyo, Japan).

Results and Discussion

The patterned immobilization of insulin using a photomask (Figure 2a) was observed by immunofluorescene staining by anti-insulin antibody as shown in Figure 2b. CHO-T cells were cultured on the film immobilized with insulin in a prescribed pattern in DMEM in the absence of serum. The cells adhered on the film independently of the immobilized insulin after 8 hours of culture (Figure 2c). However, only the cells on the immobilized insulin regions were stained by the anti-phosphotyrosine antibody (Figure 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 Ito *et al.*[10] reported that immobilized insulin activated insulin receptors, insulin receptor substrate-1 and phosphatidylinositol-3 kinase for a longer time than native insulin. Therefore, even after 8 h of culture activated cells were observed on the insulin-immobilized regions. The phenomenon was considered to be due to inhibition of receptor down-regulation by internalization or inhibition of the potential interaction of receptor with regulators of receptor activity such as tyrosine phosphatases and serine/threonine-dependent protein kinases by prevention of lateral diffusion of activated receptor in the plane of the membrane.

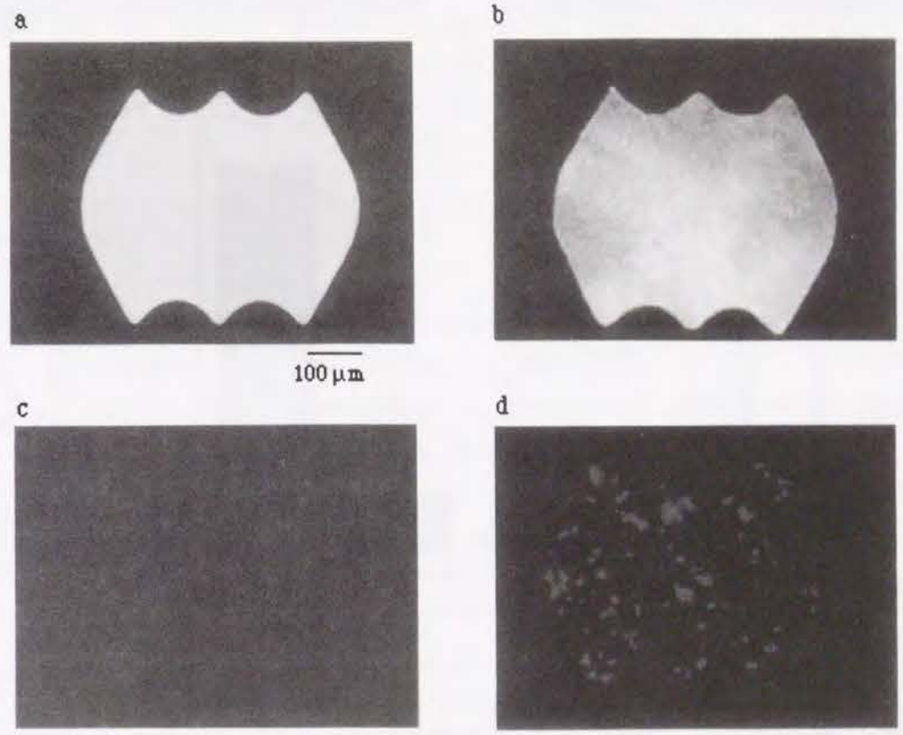

Figure 2 Micrographs of photomask (a), immunofluorescence staining of immobilized insulin with anti-insulin antibody (b), optical micrograph of adhered CHO-T cells after 8-h culture (c), and immunofluorescence staining of the CHO-T cells with anti-phosphotyrosine antibody (d).

Figures 3a and 3b show that only the cells on the regions immobilized with insulin grew in the medium containing no serum. The enhancement of cell growth was

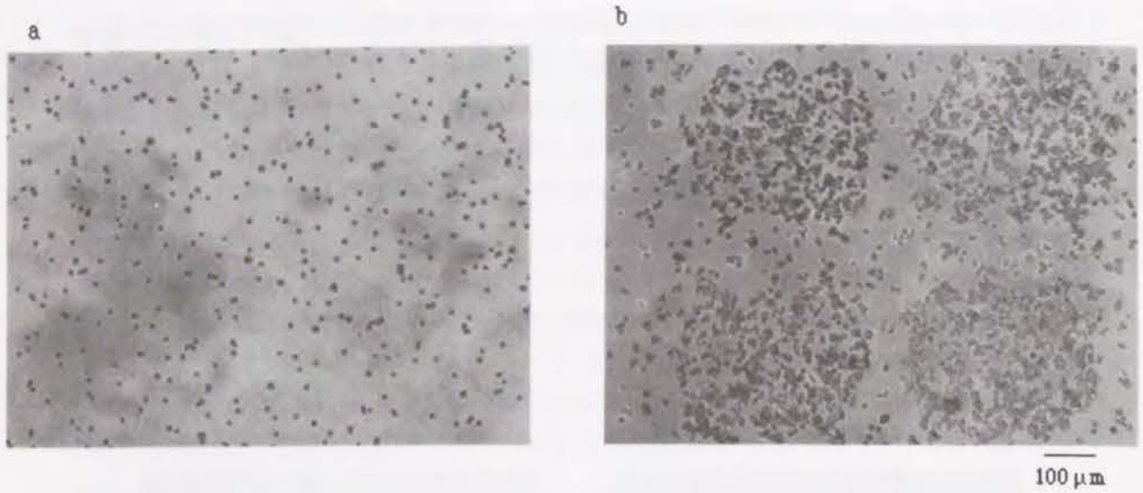

Figure 3 Phase-contrast micrographs of CHO-T cells cultured on the film immobilized with insulin in a prescribed pattern as shown in Figure 2 before (a) and after culture for 3 days (b). The cell densities in the regions with immobilized and the control regions before and after cultivation (c). n=10. Bars represent the standard deviations.

considered to be the result of signal transduction. Figure 3c compares the cell densities on the regions immobilized with insulin and the control regions of non-immobilized insulin before and after culture for three days. The increase in the number of cells on the

immobilized insulin regions was not caused by cell migration from the non-immobilized regions to the immobilized regions. No significant cell growth was observed on the control regions of untreated poly(ethylene terephthalate) film.

Although the immobilized biosignal is an important technique for elucidation of the biosignaling mechanism and molecular design of drugs or medical materials, it has not sufficiently examined after the pioneering investigation using sepharose gel with immobilized insulin[11,12]. The stimulation of the growth of only the cells on the immobilized insulin regions indicated that diffusible insulin was absent in the culture system. Recent studies on overlapping of adhesion molecules and growth factors[13], the growth stimulation by noninternalizing EGF[14], and various juxtacrine stimulators[15,16], suggest that the signal of immobilized insulin was transduced and cell growth was stimulated without internalization. By similar methods it will be possible to manipulate cells and tissues by artificial substrata with covalently immobilized growth factors and cytokines.

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Chapter 8

Artificial Juxtacrine Stimulation by Micro-Patterned Immobilization of Epidermal Growth Factor

Introduction

Intercellular interactions trigger changes of cellular function and various biological phenomena. These interactions are mediated by proteins of two different kinds, growth factors and adhesion factors. Growth factors are generally secreted as diffusible proteins and transduce the signal either towards proliferation or towards differentiation, while adhesion factors are linked to extracellular matrices and assemble up animal cells to tissues. However, these two communication systems are substantially overlaping[1-3]. For example, integrins act as receptor molecules for cell-adhesion proteins and transduce signals from the extracellular matrix by binding to the cytoskeleton [4-6].

In addition, several membrane-anchored proteins such as growth factors and lymphokines are biologically active as transmembrane "juxtacrine stimulators". These include a family of EGF [7] including transforming growth factor-α [8,9], tumor necrosis factor-α (TNF-α)[10], colony-stimulating factor-1[11], and the c-kit ligand (KL) [12-15], as reviewed by Massague and Pandiella[16]. The biological importance of transmembrane KL has been demonstrated *in vivo*. Schmid *et al.*[17] have shown that a coculture system of human umbilical cord vein endothelial cells (HUVECs) and CHO transfectants expressing a noncleavable, exclusively membrane-bound form of TNF-α potently activates HUVECs with the synergestic action of two TNF receptors. The mitogenic effect of heparin-binding EGF-like growth factor has been shown by Higashiyama *et al.*[18]. Interleukin-1 stimulates cells by means of a juxtacrine mechanism[19,20]. These mechanisms were deduced from studies of intercellular regulation by paraformaldehyde-fixed cells that express the growth factors or cytokines.

Recently, artificial juxtacrine stimulation has been achieved by several groups. Horwitz *et al.*[21] showed that interleukin-2 covalently immobilized on a polystyrene plate maintained the viability of an interleukin-2-dependent cell line. It was shown that immobilized growth factors such as EGF and insulin enhanced the growth of anchorage-dependent cells, including mouse fibroblast STO cells[22-24], bovine endothelial cells[25], and mouse sarcoma cells[26]. Ito *et al.*[27] also demonstrated that, although the time required for immobilized insulin to stimulate insulin receptors of CHO cells was longer than that

required for native insulin, receptor activation persisted longer with the immobilized ligand than with free insulin.

In this study, mouse EGF was immobilized in a prescribed micro-pattern on a polystyrene plate, and the mitogenic effect and activation of signal proteins were investigated using CHO cells that overexpress EGF receptors (CHO-ER cells).

Materials and Methods

Materials

Anti-EGF IgG was purchased from Becton Dickinson Labware (Bedford, MA).

Preparation of Photo-Reactive Polyallylamine and Photo-Reactive EGF

The photo-reactive polyallylaimne and photo-reactive EGF was synthesized as described in chapter 4.

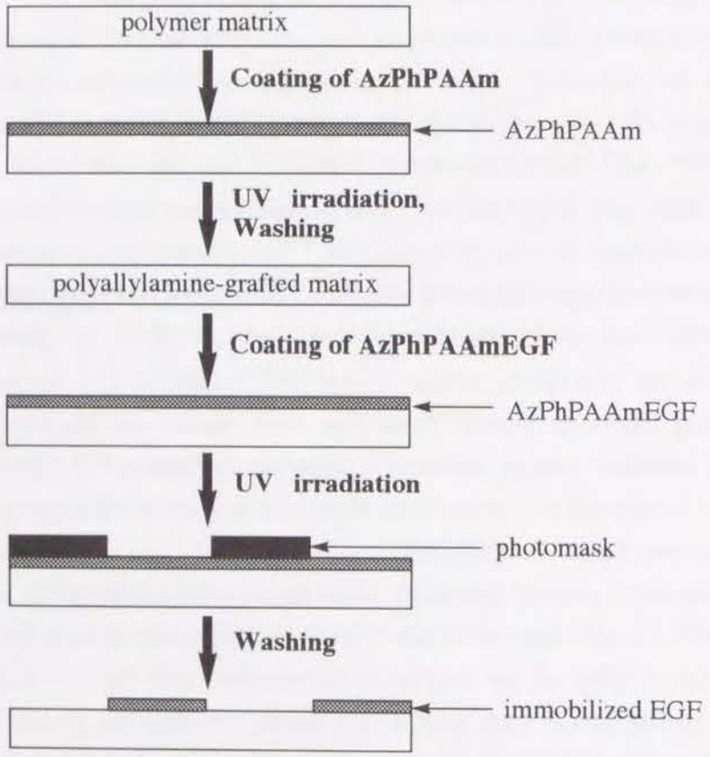

Figure 1 Microprocessing of immobilization of EGF in a specific micro-pattern on a polystyrene plate.

Microprocessing

EGF was immobilized on a polystyrene plate as shown in Figure 1. An aqueous solution of AzPhPAAm (200 μg/mL, 100 μL) was cast on a PSt plate in the shape of circle (diameter of 10 mm) and air-dried at room temperature. Then the plate was irradiated using a UV lamp (Koala, 100 W) from a distance of 5 cm for 10 s. The plate was thoroughly washed with diluted hydrochloric acid (pH = 3.0) until the absence of released AzPhPAAm was confirmed by ultraviolet absorbance at 280 nm (7 days).

Subsequently, the photo-reactive EGF conjugate (200 μg/mL, 100 μL) was cast on the PAAm-grafted PSt plate and air-dried at room temperature. The plate was covered with a photomask of a specific pattern and irradiated with the ultraviolet lamp from a distance of 5 cm for 10 s. Finally, the plate was washed with PBS at 4 ℃ until the absence of released EGF was confirmed by ultraviolet absorbance at 280 nm (7 days).

Observation of Micro-Pattern of EGF by Immunofluorescence Micoscopy

The patterned plate was stained by anti-EGF IgG antibody and observed with a fluorescence microscope as described in chapter 7.

Cell Culture

CHO-ER cells (2.5×10^5 receptor molecules per cell) were subcultured in Ham's F-12 medium containing 10% (v/v) FBS under a 5% CO_2 atmosphere at 37℃. The subcultured CHO-ER cells were harvested with PBS(-) containing trypsin (0.15% w/v, 2,000 unit/g) and EDTA (0.02% w/v). After washing once with Ham's F-12 medium containing FBS and once with Ham's F-12 medium, the cells were suspended in Ham's F-12 medium (1×10^6 cells/mL). The cell suspension was added to a 6-well tissue culture plate (0.1 mL/well) containing the patterned PSt plate that had been incubated in the well in the presence of Ham's F-12 medium (5 mL) at 37 ℃ for 2 h. The cells were cultured under a 5% CO_2 atmosphere at 37 ℃ for 48 h and observed with a phase-contrast microscope.

Observation of Cells by Immunofluorescence Microscopy

The CHO-ER cells were stained by anti-phosphotyrosine mouse IgG and observed by a laser fluorescence microscope as described in chapter 7.

Results and Discussion

Patterned Immobilization of EGF

Micro-patterned immobilization of EGF was observed by immunofluorescence staining with anti-EGF antibody (Figure 2).

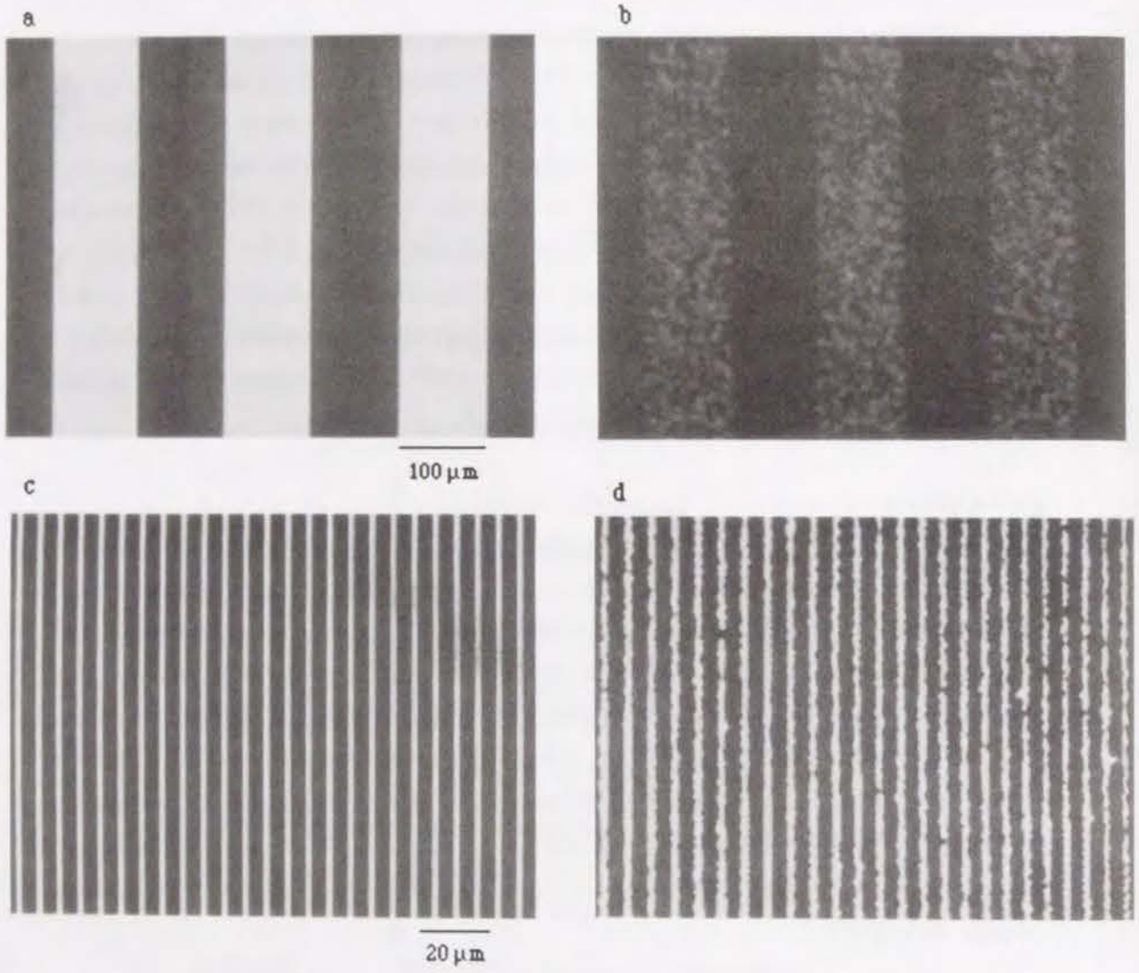

Figure 2 Micrograph of photomask with a 100-μm pattern (a), fluorescence micrograph of EGF immobilized in a stripe pattern with 100-μm width (b), photomask with a 2-μm pattern (c), and fluorescence micrograph of EGF immobilized in a stripe pattern with 2-μm width (d).

After culturing on immobilized EGF for 30 min, CHO-ER cells were stained with anti-phosphotyrosine mouse IgG and rhodamine-conjugated anti-mouse IgG

antibodies. Figure 3a shows the uniform adherence of cells on the surface of patterned plate, indicating the absence of enhancement of cell adhesion by immobilized EGF. However, Figure 3b shows that the phosphorylated tyrosine residues were detected only in the cells adhered on the EGF-immobilized regions, indicating a signal transduction occurring only through immobilized EGF.

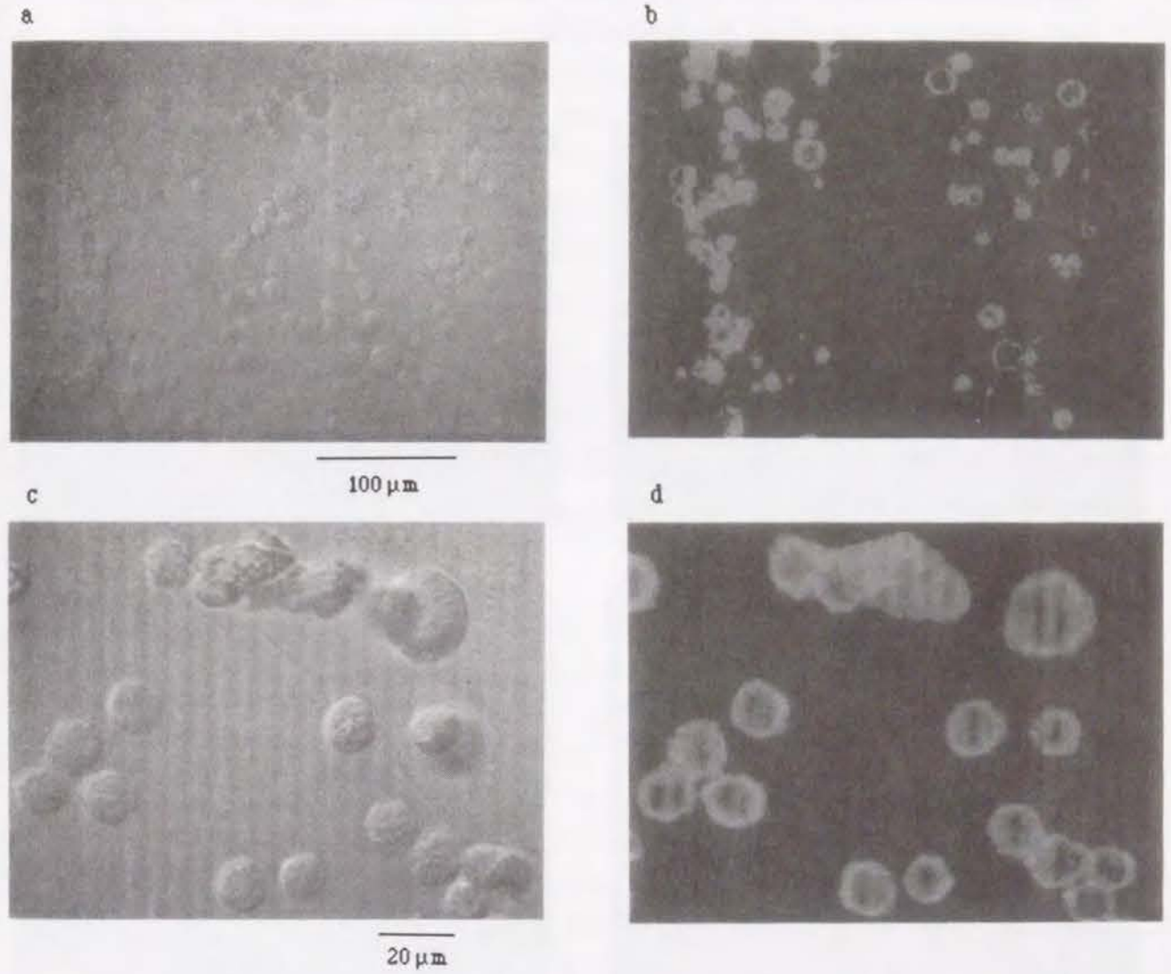

Figure 3 Phase-contrast micrograph of CHO-ER cells cultured for 30 min on a polystyrene plate having EGF immobilized in a 100-μm pattern (a), immunofluorescence staining of CHO-ER cells with anti-phosphotyrosine antibody (b), phase-contrast micrograph of CHO-ER cells cultured for 30 min on a polystyrene plate having EGF immobilized in a 2-μm pattern (c), and immunofluorescence staining of CHO-ER cells with anti-phosphotyrosine antibody (d).

Figures 3c and 3d show the cells adhering on the EGF immobilized in a stripe pattern with a 2-μm width. Only the tyrosine residues on the cell surface that contacted with the stripe regions immobilized with EGF in 2-μm width were stained by anti-phosphotyrosine antibody. Since the free lateral diffusion and internalization of bound EGF/EGFR complex are prohibited by immobilization of EGF, a partial activation of signal proteins resulted. These results indicate that the biological signal is transduced to the cells only that interact with immobilized EGF.

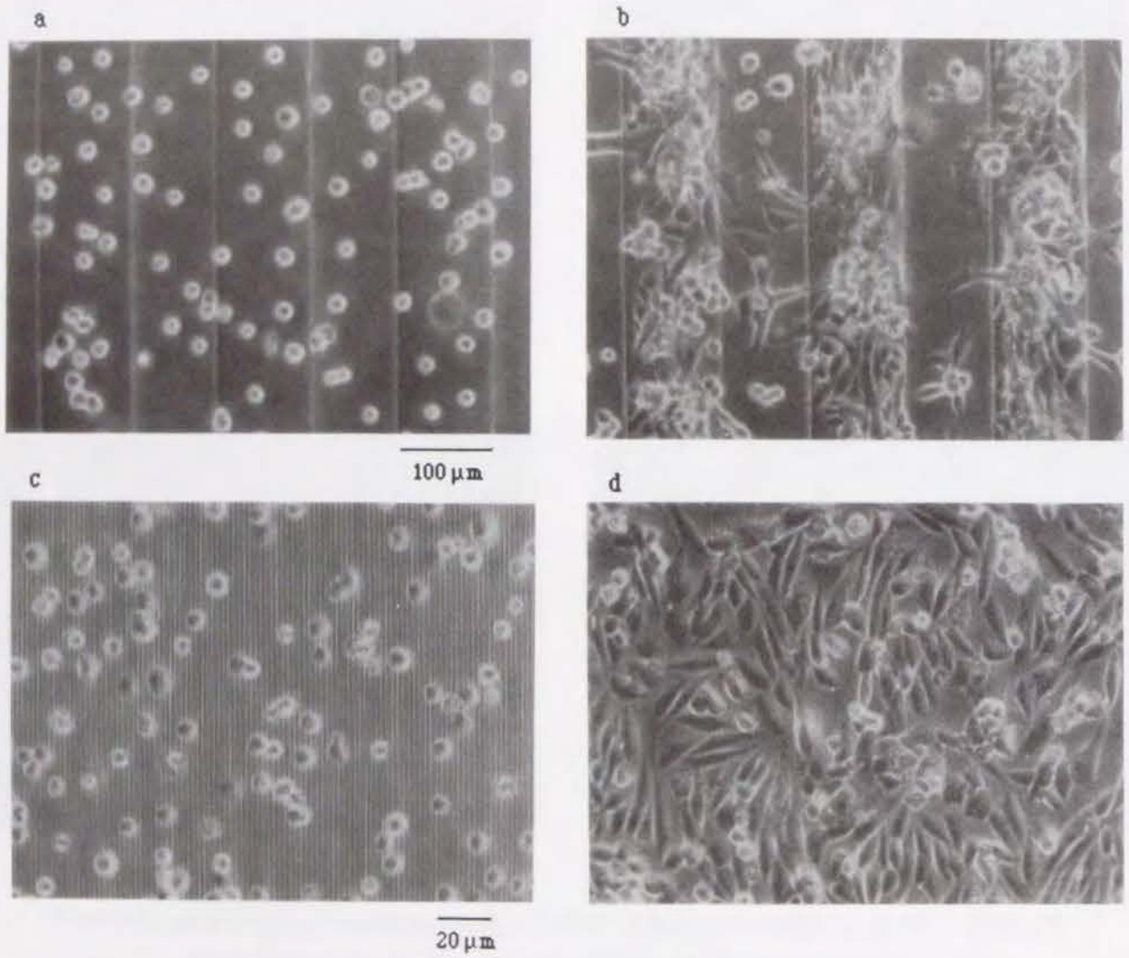

Figure 4 Phase-contrast micrographs of CHO-ER cells on EGF immobilized in a stripe pattern of 100-μm width before (a) and after 48-h culture (b), and on a plate having EGF immobilized in a stripe pattern of 2-μm width before (c) and after 48-h culture (d).

Cell Growth on the Micro-Patterned EGF

CHO-ER cells before and after 48-h culture on the culture plate with EGF immobilized in a micro-pattern (100-μm width or 2-μm width) are shown in Figure 4. When the width of stripe pattern is bigger (100-μm width) than the size of cell, patterned growth of cells was observed as shown in Figure 4b. However, when the pattern width is smaller than the size of cell (2-μm width), patterned growth did not occur (Figure 4d). Therefore, the patterned growth as observed in Figure 4b should have been caused by the enhancement of cell growth due to signal transduction from the immobilized EGF.

Taking into consideration of the results obtained from immobilized insulin as described in chapter 7, the immobilized growth factors should stimulate the cells without internalization. In future, it would be interesting to immobilize various kinds of growth factor and to investigate the effect by the present methodology.

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CONCLUDING REMARKS

This thesis summarizes the results of investigations on the chemical and biological modifications of the surfaces of polymer matrices by immobilizing thermo-responsive polymer or cell-growth factors such as insulin and EGF. Cell culture on the chemically or biologically modified matrices resulted in regulation of cell behaviors such as adhesion, detachmentt, growth, differentiation, and tissue construction.

In Part I of the present thesis, surface biolization of polymer film by immobilization of cell-growth factor, insulin or EGF, for enhancement of growth and promotion of differentiation was investigated.

In chapter 1, insulin was covalently bound to water-soluble polymers such as POE and PAA. These water-soluble insulin derivatives are monovalent and multivalent insulin conjugates, respectively. Also, insulin was immobilized to a PAA-grafted PSt film to form a water-insoluble multivalent conjugate. The water-soluble monovalent insulin conjugate showed lower mitogenic activity than the native insulin, but the water-soluble multivalent insulin conjugate led to faster cell growth than the native insulin. Much lower concentration of immobilized insulin than the native insulin and the water-soluble insulin conjugates brought about similar acceleration of cell growth. The maximal mitogenic effect of the immobilized insulin was greater than that of native insulin or the water-soluble insulin conjugates. These findings suggest that the greater acceleration of cell growth by the immobilized insulin comes from long-lasting mitogenic effect owing to the absence of internalization into the cell.

In chapter 2, insulin was photo-immobilized on a culture plate made of PSt. The photo-immobilized insulin enhanced the growth of anchorage-dependent cells such as Chinese hamster ovary CHO-K1 cell and mouse fibroblast STO cell more strongly than the native insulin or azidophenyl-derivatized insulin which is a synthetic intermediate. A small amount of photo-immobilized insulin enhanced the growth of CHO-K1 and STO cells. In addition, the maximal mitogenic effect of the immobilized insulin was greater than that of the native insulin or the derivatized insulin.

In chapter 3, EGF was photo-immobilized on a culture plate of PSt. The photo-immobilized EGF enhanced the growth of anchorage-dependent cells more extensively than the native EGF or the azidophenyl-derivatized EGF. A small amount of photo-immobilized EGF was sufficient to enhance the growth of cells and the maximal mitogenic effect was greater than that of native EGF or the derivatized EGF. On the other hand, the photo-immobilized EGF did not enhance growth of anchorage-independent cells. These results imply that the enhanced cell growth results from interaction of the cells with

the immobilized EGF. Photo-immobilization is a useful means to fix growth factors onto a polymer film that is devoid of functional groups to connect growth factors and also to elucidate the mechanism of signal transduction. The growth-factor-immobilized film will constitute a new protein-free cell culture system.

In chapter 4, it was demonstrated that the immobilized EGF did not enhance cell growth but promoted differentiation of PC12 cell to neural cell. The immobilized EGF activated MAP kinase for a longer time than native EGF and promoted translocation of activated MAP kinase from cytosol to nucleus. In addition, the immobilized EGF activated the neurite formation of PC12 cell like NGF. It is known that NGF, which promotes PC12 cell to neuronal differentiation, activates MAP kinase for a longer time than native EGF, which stimulates PC12 cell to cell proliferation. The difference of MAP kinase activation could be related with switching PC12 cell between growth and differentiation. The present study demonstrated that the growth-differentiation switching depended on the kinetics of MAP kinase activation. The switching can be regulated by immobilization of EGF.

In Part II of this thesis, thermal control of cell adhesion and detachment to/from the surface of polymer film was investigated by using a hybrid polymer film having a thermo-responsive polymer grafted.

In chapter 5, a thermo-responsive polymer, poly(*N*-isopropylacrylamide) (PNIPAAm), was grafted in a prescribed pattern on a PSt plate by photo-lithography. The surface wettability of regions grafted with PNIPAAm was variable with temperature, although that of ungrafted regions was independent of temperature. The surface of PNIPAAm-grafted regions was hydrophobic at 37 ℃, but hydrophilic at 4 ℃. The micro-pattern of the graft chains on the surface was clearly observed at 37 ℃ by phase-contrast microscopy. The micro-pattern disappeared by lowering temperature because of hydration of the graft chains. The PSt plate with the micro-patterned thermo-responsive graft chains was employed for cell culture. Mouse fibrobalst STO cells that attached onto the PSt plate at 37 ℃ detached selectively from the regions grafted with PNIPAAm by lowering temperature.

In chapter 6, insulin was coupled with a polymer film on which a thermo-responsive polymer was grafted in advance. The LCST of the thermo-responsive polymer, poly(*N*-isopropylacrylamide-*co*-acrylic acid), was not significantly affected by the insulin coupling. The thermo-responsive polymer was grafted to glow-discharged polystyrene film and insulin was covalently coupled (immobilization). The surface wettability of the insulin-immobilized film was high at a temperature lower than LCST but low at a temperature higher than LCST. The immobilized insulin exhibited a very strong mitogenic

activity as described in chapter 1. About half of the viable cells were detached from the film only by lowering temperature. The recovered cells proliferated normally on a new culture dish.

In Part III of the thesis, geographic control of biomaterials function for micro-patterning of cell proliferation was investigated.

In chapter 7, photo-reactive PAA derivative was synthesized and grafted on a poly(ethylene terephthalate) film in a prescribed pattern, and then insulin was covalently immobilized on the regions where PAA was photo-grafted. The patterned immobilization of insulin was confirmed by immunofluorescence staining with anti-insulin antibody. Only CHO-T cells which adhered on the regions immobilized with insulin were stained with the anti-phosphotyrosine antibody, indicating that the immobilized insulin transmitted growth signal to the cells through phosphorylation of tyrosine residues of signal proteins. The cells proliferated in the same pattern as the immobilized insulin.

In chapter 8, EGF was immobilized on a PSt plate in a prescribed micro-pattern. The immobilized EGF transmitted biological signal to CHO-ER cells through phosphorylation of signal proteins and proliferated the cells. The micro-patterned immobilization critically demonstrated the effect of immobilized EGF. Patterned stimulation of signal transduction proteins by the immobilized EGF in a width smaller than the adhered cells was also observed. The results of chapters 7 and 8 clearly indicated the interaction between immobilized growth factors and cells.

Chemical/biological modification of the surface of a polymer film with signal-responsive polymers or biological proteins is a powerful tool to construct elaborate biomaterials. The control of surface functionality by micro-patterned immobilization of these proteins is a skilled tool either to investigate the mechanism of cellular signal transduction or to realize precise control of cell behaviors on the biomaterial matrices.

LIST OF PUBLICATIONS

Chapter 1 Mitogenic Activities of Water-Soluble and -Insoluble Insulin Conjugates
Guoping Chen, Yoshihiro Ito and Yukio Imanishi
Bioconjugate Chem., in press.

Chapter 2 Photo-Immobilization of Insulin onto Polystyrene Dishes for Protein-Free Cell Culture
Yoshihiro Ito, Guoping Chen and Yukio Imanishi
Biotechnol. Prog., *12*, 700 (1996).

Chapter 3 Photo-Immobilization of Epidermal Growth Factor Enhances Its Mitogenic Effect by Artificial Juxtacrine Signaling
Guoping Chen, Yoshihiro Ito and Yukio Imanishi
Biochim. Biophys. Acta, accepted.

Chapter 4 Switching from Growth to Differentiation in PC12 Cell by Immobilization of Epidermal Growth Factor
Yoshihiro Ito, Guoping Chen, Yukio Imanishi, Yoshinori Okabayashi and Masato Kasuga
Nature, submitted.

Chapter 5 Patterned Immobilization of Thermo-Responsive Polymer
Yoshihiro Ito, Guoping Chen, Yanging Guan and Yukio Imanishi
Langmuir, accepted.

Chapter 6 Regulation of Growth and Adhesion of Cultured Cells by Insulin Conjugated with Thermo-Responsive Polymers
Guoping Chen, Yoshihiro Ito and Yukio Imanishi
Biotechnol. Bioeng., ***53***, 339 (1997)

Chapter 7 Patterned Artificial Juxtacrine Stimulation of Cells by Covalently Immobilized Insulin
Yoshihiro Ito, Satoshi Kondo, Guoping Chen and Yukio Imanishi
FEBS Lett., accepted.

Chapter 8 Artificial Juxtacrine Stimulation by Micro-Patterned Immobilization of Epidermal Growth Factor
Yoshihiro Ito, Guoping Chen and Yukio Imanishi
J. Biol. Chem., submitted.

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