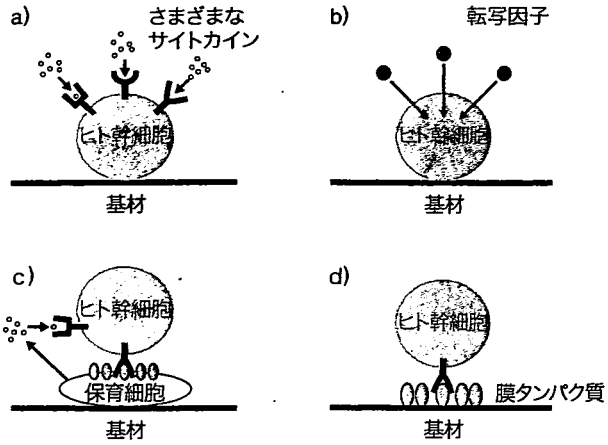

図3 幹細胞培養のためのバイオリアクター設計
a) さまざまなサイトカインを組み合わせて培養液に添加，b) 細胞内移行性の転写因子タンパク質を培養液に添加，c) 保育細胞をあらかじめ培養して，そのうえで培養，d) 保育細胞の発現する膜タンパク質を固定化したうえで培養．

胞培養用のバイオリアクターである．造血幹細胞はもともと血液細胞の源となるもので，成人では骨髄のなかでつくられる．そこで，骨髄中の環境を再現しようとさまざまな試みが行われ，これまでさまざまなタイプのバイオリアクターが開発されてきている[2]．近年では，とくに骨髄内部の環境（ニッチェ）が重要な役割を果たしていることから，ナノスケールでその環境を再現することが必要と考えられ，三次元構造のマトリックスのなかで細胞を培養するバイオリアクターがいろいろと考案されている[3]（図3）．培養液に添加する物質についても，サイトカインだけでなく，造血幹細胞の成長を促進するような転写因子タンパク質に細胞内移行ペプチドをつけたナノデバイスを作用させるような試みも行われている．

最も効果的な方法に，ナース（保育）細胞やフィーダー（供給）細胞と呼ばれる細胞と共培養することによりニッチェを調製することがある．当初は生物学的な研究が主で，マウスのような実験動物由来の細胞を保育細胞としていたが，不死化させたヒト由来の骨髄ストローマ細胞が造血幹細胞増幅支持能をもつことが明らかになり，造血幹細胞を定常的に培養することが可能になった．最近では，この増殖支持能が培地に栄養分や増殖因子を補給するフィーダー作用としてだけではなく，直接の細胞間の接触によってその機能を発揮していることがグルタルアルデヒドのような物質で骨髄ストローマ細胞を化学的に固定化しても活性を保持することから明らかにされた[4]．ES細胞培養にも一般的に保育細胞が必要であることが知られているが，この場合にも同様な処理が有効であることがわかってきた．

課題となるバイオリアクターナノ界面の開発

このように骨髄の環境を再現するためには必ずしも生きた細胞でなくてもよいことがわかってきたが，細胞を使って基材をつくるのは，大量生産が困難なうえに，病原体の混入の危険は避けられない．もし完全に人工的な素材で幹細胞保育基材をつくることができれば，再生医療の本格的な実現のための大きな一歩となる．今まで人工的に保育細胞を模倣するような基材の開発は行われてこなかった．これは今後解決していかなければならない重要な課題となっている．

数少ない解決策として現在取り組まれているものの一つが，成長因子やサイトカインのようなタンパク質や，新たに保育細胞から増幅支持能に関与する膜タンパク質を探しだ

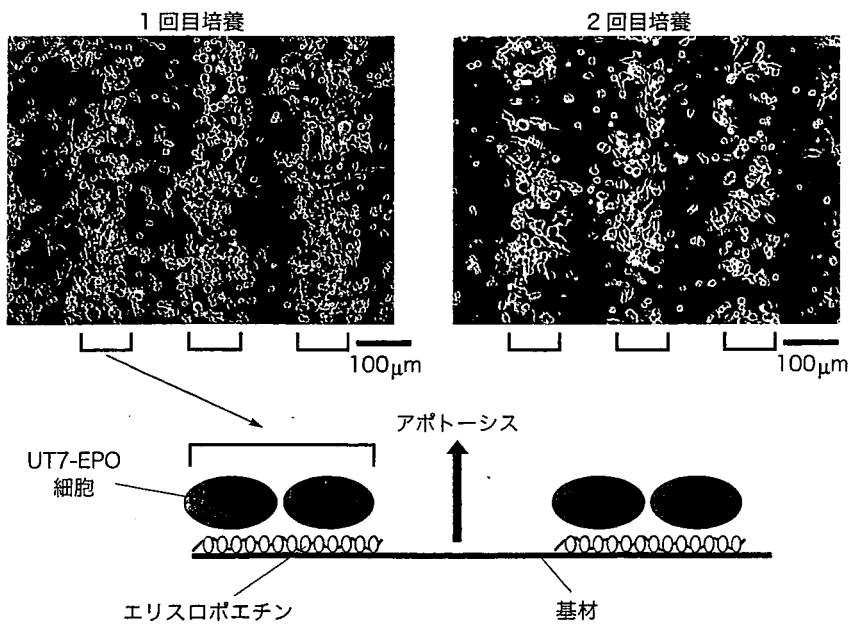

図4 エリスロポエチン固定化基板上でのエリスロポエチン依存性ヒト白血病由来細胞の培養
ストライプ状にエリスロポエチンが固定化されており，固定化領域でのみ細胞増殖が観測されている．また，1回培養に用いた固定化エリスロポエチンを2回目の培養に用いることも可能であった．

し，これらを固定化した材料を開発することである．これまでに，インスリン，上皮細胞成長因子，神経成長因子，腫瘍壊死因子，血管内皮細胞成長因子，肝細胞成長因子，骨形成タンパク質などを固定化して細胞機能を制御できることが多くの研究者により明らかにされてきた[5]．図4には，モデル細胞としてエリスロポエチン（EPO）依存性のヒト白血病由来細胞を選び，サイトカインのEPOをマイクロパターン状に固定化したうえで培養した結果を示す．3日間経過するとEPOが固定化されていない表面では細胞はアポトーシスを誘起され死滅したが，固定化されている領域では，増殖が観測された．これは固定化EPOがこの細胞に特異的に働き，アポトーシスを抑制したものと考えられる．また，培養した細胞をいったん剥離し，再度新しい細胞を播種して培養しても，EPO固定化領域でのみ増幅が観測された．酵素を固定化したバイオリアクターと同じような使い方ができることが明らかになった．

また，膜タンパク質として知られるNotchリガンドを固定化した場合でも，固定化領域でだけ依存性細胞の接着，増殖が観測されている．このほか，マウスES細胞培養では，通常の培養で必要となる白血病阻害因子（leukemia inhibitory factor；LIF）を固定化し，その上でマウスES細胞を培養すると未分化を維持したまま増幅できることも報告されている．また，細胞間接着タンパク質のカドヘリン固定化材料は新しい機能を発揮することが見いだされている[6]．

ただ残念ながら，まだ実用的に臍帯血造血幹細胞や，そのほかの有用な細胞を効果的に体外増幅できるところまでには至っていない．現在知られているサイトカインや膜タンパク質を組み合わせて，幹細胞培養用の人工ニッチェを構築することは不可能なようである．どのようなタンパク質を，何種類，どのくらい，どのような組合せで基材上にナノテクノロジーで再構築したらよいのか（図5）？　生命科学の分野では未知の（膜）タンパク質の探索が精力的に行われている．文部科学省「再生医療の実現化プロジェクト」の一環で，臍帯血造血幹細胞の体外増幅に役立つ方策の募集も行われている．細胞培養には幹細胞に限らず，保育細胞が必要な場合が多く，この課題解決への期待は大きい[7]．

これまで細胞培養基材については接着抑制や促進の制御が限界であり，それ以上の細胞の高次機能（成長や分化など）を制御するような基材の開発にはなす術もなかった．今後明らかになることが期待されるタンパク質を用いて，細胞機能を制御できるナノ界面を基材上に生みだせるようにすること

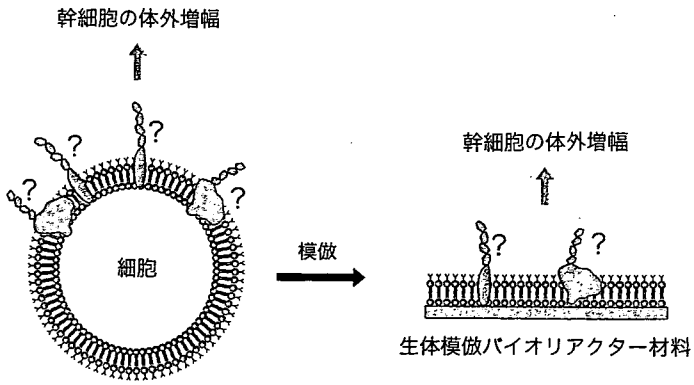

図5　実現が望まれる幹細胞体外増幅用のナース細胞を模倣したナノ界面をもつ人工基材

が，バイオマテリアル研究の大きな課題といえる．

●

日本の幹細胞の生物学的研究は世界をリードする状況にある．体性幹細胞を用いた再生医療は着実に発展してきている．日本でも基礎研究に根ざすさまざまな可能性を生かした臨床応用への展開研究であるトランスレーショナル研究が重要視されるようになっている．本誌『化学』の若い読者が基礎的な化学のトレーニングを受けたあと，この特集で取りあげられているナノメディシンのような新しい研究分野でぜひ力を発揮してもらいたい．

謝　辞

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Chondrogenic differentiation of human mesenchymal stem cells on photoreactive polymer-modified surfaces

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Abstract

Human mesenchymal stem cells (MSCs) were cultured on polystyrene surfaces modified with photoreactive azidophenyl-derivatives of three different chargeable polymers, poly(acrylic acid) (PAAc), polyallylamine (PAAm), and poly(ethylene glycol) (PEG). The MSCs adhered and spread both on a PAAm-modified surface and on PAAc-modified and polystyrene (control) surfaces. However, the cells adhered more easily to the PAAm-modified surface. The MSCs did not attach to the PEG-modified surface and aggregated to form pellets immediately after cell seeding. The cells proliferated on the PAAc-, PAAm-modified and control surfaces with culture time, formed a monolayer, and aggregated to form pellets. The cells in the pellets that formed on the PAAm- and PEG-modified surfaces after 2 weeks culture had a round morphology and the extracellular matrices were positively stained by safranin O and toluidine blue, while those that formed on the PAAc-modified and control surfaces had a spindle, fibroblast-like morphology and were not positively stained by safranin O and toluidine blue. The pellets that formed on the PAAm- and PEG-modified surfaces contained significantly higher levels of sulfated glycosaminoglycans than did those that formed on the PAAc-modified and control surfaces. Type II collagen and cartilage proteoglycan were immunohistologically detected in the pellets that formed on PAAm- and PEG-modified surfaces, but not those that formed on the PAAc-modified and control surfaces. The MSCs cultured on the PAAm- and PEG-modified surfaces expressed a high level of cartilaginous genes encoding type II collagen and aggrecan, while the MSCs cultured on the PAAc-modified and control surfaces did not express these genes. These results suggest that the PAAm-modified surface supported cell adhesion and proliferation and also promoted chondrogenic differentiation of the MSCs. The PAAc-modified and polystyrene surfaces supported cell adhesion and proliferation, but not chondrogenic differentiation. The PEG-modified surfaces did not support cell adhesion, but did promote chondrogenic differentiation. The adhesion, proliferation, and differentiation of the MSCs could be controlled by surface chemistry.

Keywords: Mesenchymal stem cells; Chondrogenic differentiation; Surface modification; Surface grafting; Surface property

1. Introduction

The surface properties of biomaterials and scaffolds such as chemical composition, nano- or microstructured morphology, wettability, and electrostatic property are very important for cell behaviors such as cell attachment, proliferation, extracellular matrix (ECM) secretion, and differentiation [1–5]. To elucidate the effects of surface properties, especially surface chemistry on cell functions, various methods have been reported to present the surfaces with different functional groups.

Poly(ethylene glycol) (PEG)-terephthalate–poly(butylene terephthalate) (PEGT/PBT) block copolymer substrates with various PEG lengths and mole fractions have been used to study the surface properties such as wettability, swelling, biodegradation rate and mechanical properties on

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protein adsorption and chondrocyte functions [6–8]. The block copolymer substrates with high PEG molecular weight and PEGT ratio exhibited a low fibronectin to vitronectin adsorption ratio and resulted in poor cell attachment, but showed an increased ability to maintain the primary human articular chondrocyte phenotype. In contrast, those with low PEG molecular weight and PEGT ratio showed preferential surface adsorption of fibronectin compared to vitronectin and enabled cell attachment, but resulted in chondrocyte dedifferentiation to a fibroblastic phenotype. A balance of hydrophilic and hydrophobic segments is needed for chondrocyte attachment and maintenance of the chondrogenic phenotype.

Self-assembled monolayers (SAMs) of alkanethiols on gold are another useful model system to systematically investigate the effects of surface chemistry on cell functions. Many researchers have used this method to study the functional surface groups on the adsorption and conformational change of proteins and the resulting effects on cell adhesion, spread, alignment, and proliferation [9–14]. Methyl, hydroxyl, carboxyl, and amino groups have been presented on gold surfaces by SAMs to study their effects on cell functions in different culture conditions. The effects depend not only on surface composition, but also on culture condition and cell type.

Silane-modified glasses presenting methyl ($-CH_3$), hydroxyl (–OH), carboxyl (–COOH), amino ($-NH_2$), and silane (–SH) have also been used. Curran et al. [15,16] reported the effects of surfaces presenting these functional groups on the differentiation of human mesenchymal stem cells (MSCs). The glass control and $-CH_3$ surfaces maintained the multipotent phenotype of MSCs, the $-NH_2$- and –SH-modified surfaces promoted osteogenesis, and the –OH- and –COOH-modified surfaces promoted chondrogenesis.

The above-mentioned methods of surface modification can create a well-defined surface chemistry, but the use of copolymers is limited to specific copolymers such as PEGT/PBT. The SAM method and silane-modification are only applicable to gold and glass substrates. Matsuda and Ito have developed a method of photochemical modification that can be used to introduce functional groups to the surfaces of any organic substrate [17–19]. The introduced groups are covalently bound to the surface and remain stable during long-term cell culture. In this study, we used this method for the surface modification of polystyrene cell culture plates. Three kinds of surfaces with different functional groups/electronic properties were designed and prepared by the photochemical method, namely a NH_2/positively charged surface, which was modified by photoreactive polyallylamine (PAAm), a COOH/negatively charged surface, which was modified by photoreactive poly(acrylic acid) (PAAc), and a neutral surface, which was modified by photoreactive PEG. The functional groups were grafted on the surface of polystyrene cell culture plates and their effects on the adhesion, proliferation, and chondrogenic differentiation of human MSCs were investigated.

2. Materials and methods

2.1. Synthesis of azidophenyl-derivatized poly(acrylic acid)

Azidophenyl-derivatized PAAc conjugate was synthesized by coupling PAAc with 4-azidoaniline, as shown in Fig. 1a. PAAc (Sigma-Aldrich Inc., M_w = 450,000, 1.0 mmol on monomer unit), 4-azidoaniline hydrochloride (Sigma-Aldrich Inc., 0.1 mmol), and 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (WSC, Wako Pure Chemical Industries Ltd., 6.0 mmol) were dissolved in deionized water (110 mL). The pH of the solution was adjusted to 7.0 by adding NaOH or HCl. After being stirred at 4 °C for 48 h, the reaction solution was dialyzed against MilliQ water through a seamless cellulose tube (cutoff M_w, 12,000) until the absence of azidoaniline in the washing solution was confirmed by ultraviolet spectroscopy. The dialyzed polymer was freeze-dried. The azidophenyl-derivatized PAAc was referred to as AzPhPAAc. The amounts of the azidophenyl groups in the polymer were determined by ^{1}H nuclear magnetic resonance (^{1}H-NMR) from the peak intensities of the azidophenyl protons at 7.0 ppm, and those of the methylene and methine protons of the polymer main chain at 1.3 and 2.5 ppm, respectively.

2.2. Synthesis of azidophenyl-derivatized polyallylamine

Azidophenyl-derivatized PAAm conjugate was synthesized by coupling PAAm with 4-azidobenzoic acid (Fig. 1b and c). *N*-(4-azidobenzoyloxy) succinimide was first synthesized (Fig. 1b). A solution of dicyclohexylcarbodiimide (13.3 g, 64.6 mmol) in tetrahydrofuran (THF 50 mL) was added dropwise to a solution of *N*-hydroxysuccinimide (Wako Pure Chemical Industries Ltd., 7.43 g, 64.6 mmol) and 4-azidobenzoic acid (Tokyo Kasei Kogyo Co. Ltd., 9.57 g, 58.7 mmol) in 150 mL THF in an ice bath under stirring. After 3 h, the reaction mixture was slowly warmed to room temperature (RT) and stirring was continued overnight. The white solid that formed was filtered off, and the solvent was removed under reduced pressure. The remaining yellow residue was crystallized from isopropyl alcohol/isopropyl ether. The azidophenyl-derivatized PAAm conjugate was then synthesized by coupling PAAm with *N*-(4-azidobenzoyloxy) succinimide (Fig. 1c). An aqueous solution (pH 7.0, 10 mL) containing PAAm (Sigma-Aldrich Inc., M_w 60,000, 30 mg) was added to the DMF solution (20 mL) of *N*-(4-azidobenzoyloxy) succinimide (8.4 mg) under stirring in ice. After being stirred at 4 °C for 24 h, the solution was ultrafiltered (Millipore MoleCut, filtration cut-off below 10 kDa), washed twice with 5 mL DMF/H_2O (1/2) solution, then with 5 mL MilliQ water until the absence of *N*-(4-azidobenzoyloxy) succinimide in the washing solution was confirmed by ultraviolet spectroscopy. The azidophenyl-derivatized PAAm was referred to as AzPhPAAm. The amounts of the azidophenyl groups in the polymer were determined by ^{1}H-NMR from the peak intensities of the azidophenyl protons at 7.0 ppm and those of the methylene and methine protons of the polymer main chain at 1.3 and 2.5 ppm, respectively.

2.3. Synthesis of azidophenyl-derived poly(ethylene glycol)

Azidophenyl-derived PEG was synthesized by the reaction of bis-amino PEG (NOF Corporation, Japan, PEG, M_w 5106, 100 mg) and *N*-(4-azidobenzoyloxy) succinimide (81 mg) in chloroform under stirring overnight (Fig. 1d). The obtained product was purified from chloroform/dehydrated diethyl ether three times until the absence of *N*-(4-azidobenzoyloxy) succinimide in the reprecipitation solution was confirmed by ultraviolet spectroscopy. The azidophenyl-derivatized PEG was referred to as AzPhPEG. The amounts of the azidophenyl groups in AzPhPEG were determined by ^{1}H-NMR.

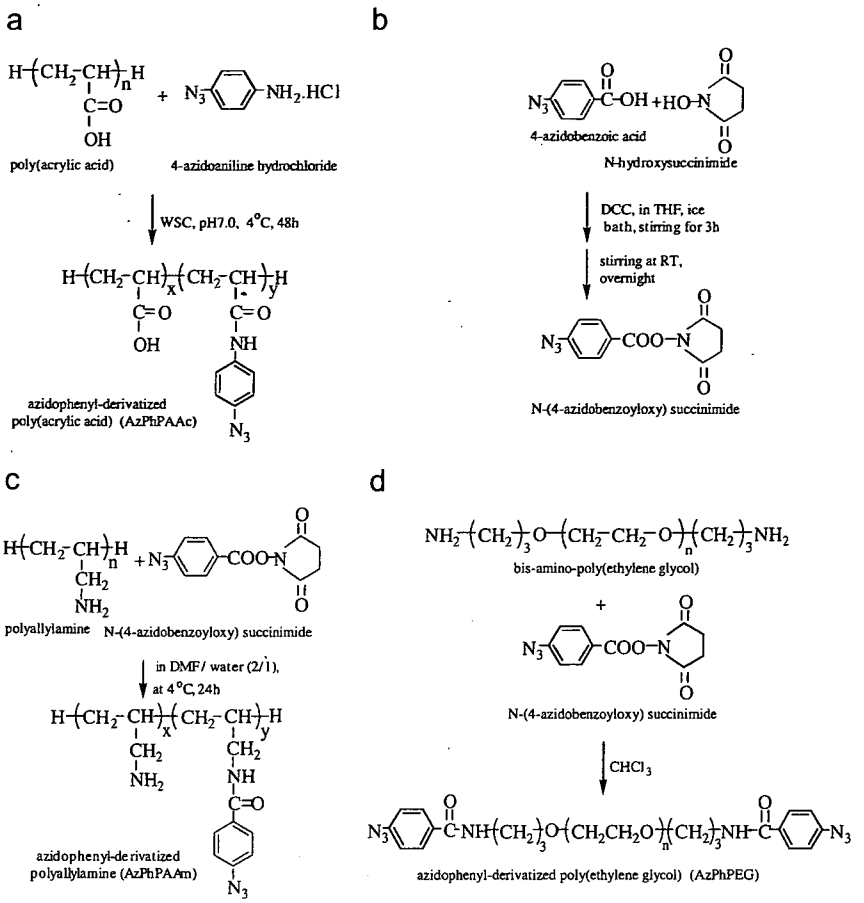

Fig. 1. Synthetic scheme of azidophenyl-derivatized poly(acrylic acid) (AzPhPAAc) (a), *N*-(4-azidobenzoyloxy) succinimide (b), azidophenyl-derivatized polyallylamine (AzPhPAAm) (c) and azidophenyl-derivatized poly(ethylene glycol) (AzPhPEG) (d).

2.4. Surface grafting of polyallylamine, poly(acrylic acid), and poly(ethylene glycol), measurement of contact angle and scanning probe microscopy (SPM) observation

AzPhPAAm, AzPhPAAc, and AzPhPEG were dissolved in water (1 mg/mL). The solutions were placed in the wells of 6-well polystyrene cell culture plates (40 μL/well) and air-dried at RT in the dark. The plates were irradiated with ultraviolet light at an intensity of 10^5 μJ/cm^2 from a distance of 15 cm for 60 s. After irradiation, the irradiated plates were immersed in diluted hydrochloric acid (pH 4), alkaline solution (pH 10), and MilliQ water, respectively and then sonicated to completely remove any unreacted polymers. After washing, the plates were sterilized with 70% ethanol aqueous solution and used for cell culture.

The contact angles of a sessile water drop on the surfaces of polymer-grafted polystyrene were measured by an Automatic Contact Angle Meter (Kyowa Interface Science Co., Ltd.). Six samples of each surface were used for the measurements. The data were expressed as the average ± standard deviation of the six samples.

For SPM observation, the AzPhPAAm, AzPhPAAc, and AzPhPEG were pattern-grafted on polystyrene discs using a patterned photomask. The AzPhPAAm, AzPhPAAc, and AzPhPEG were coated onto the polystyrene discs (4 cm^2, cut from 6-well polystyrene cell culture plates) at the same densities as those for surface grafting described above. The discs were covered with a patterned photomask having a 200 μm-wide stripe network and irradiated with ultraviolet light under the same conditions as that described above. After complete washing, the pattern-grafted surfaces were dried and observed by an SPA400 (SII NanoTechnology Inc.) equipped with an Olympus rectangular cantilever (SI-DF20) having a spring constant of 15 N/m in non-contact mode. The SPM measurements were made at RT. The thicknesses of the grafted polymers were measured from the SPM images. Nine spots from the topographic images of each kind of grafted pattern were used to calculate the mean height and standard derivatives. The data were expressed as the average ± standard deviation of the nine spots.

2.5. Cell culture

Human bone marrow-derived MSCs were obtained from Osiris (Worthington Biochemical, Lakewood, NJ) at passage 2. The cells were seeded in T-75 culture flasks using the proliferation medium from Osiris. The proliferation medium contained 440 mL MSC basal medium, 50 mL mesenchymal cell growth supplement, 10 mL 200 mM L-glutamine, and 0.5 mL penicillin/streptomycin mixture. The cells were further subcultured once after reaching confluence and used at passage 4. The cells were collected by treatment with trypsin/EDTA solution, washed once with DMEM serum-free medium, and suspended in DMEM serum-free medium at a density of 7.0×10^5 cells/mL. The cell solution was added to each well of the PAAm-, PAAc-, PEG-modified, and non-modified six-well cell culture plates (1 mL/well). Each well was supplemented with 5 mL chondrogenic induction medium and cultured for another 2 weeks under static conditions. The chondrogenic induction medium consisted of serum-free DMEM containing 4500 mg/L glucose, 584 mg/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM non-essential amino acids, 0.4 mM proline, 50 mg/L ascorbic acid, 10^{-7} M dexamethasone, and 10 ng/mL TGF-β3 (Sigma-Aldrich, St. Louis, MO, USA). The TGF-β3 was thawed and supplemented immediately before use. The medium was

changed three times per week and was done carefully to avoid removing the cell pellets that formed during culture. The cell pellets that formed after 2 weeks culture were harvested for histological examination and gene expression analysis.

2.6. Biochemical analysis

The pellets that formed after 2 weeks culture in the chondrogenic induction medium were used for biochemical analysis of DNA and sulfated glycosaminoglycans (GAGs). The pellets were washed with pure water, frozen, and freeze-dried. The dried pellets were weighed and digested with 0.5 mL of papain. The amount of DNA in the papain digests was measured with fluorescent dye, Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA), and a spectrofluorometer (JASCO, Tokyo, Japan) [20]. The sulfated GAG content was determined using a sulfated GAG assay kit, BlyscanTM (Biocolor Ltd., Newtownabbey, Northern Ireland) and an ultraviolet–visible spectrophotometer (JASCO, Tokyo, Japan) at 656 nm [21]. The amounts of DNA and GAG were divided by the weights of the pellets to determine their contents in each milligram of pellet. The GAG contents were also divided by the DNA amount to compare their contents per μg DNA. Every three pellets under each condition were used for the measurement to calculate the means and standard deviations. The data were expressed as the average ± standard deviation.

2.7. Cell proliferation

Cell proliferation was measured using the WST-1 assay (Roche Diagnostics, Indianapolis, IN, USA). This is a colorimetric assay for the quantification of cell viability and proliferation that is based on the cleavage of a tetrazolium salt (WST-1) by mitochondrial dehydrogenases in viable cells. Increased enzyme activity leads to an increase in the amount of formazan dye, which is measured with a spectrophotometer. 96-well cell culture plates were used. The three kinds of photoreactive polymers were grafted on the surfaces of the wells in the same manner as the grafting procedure described above by changing the volume of the eluted aqueous solution to 1.1 μL/well. After grafting, the plates were sterilized with 70% ethanol aqueous solution and used for cell culture. The MSC solution in DMEM serum-free medium at a density of 5.0×10^4 cells/mL was added to each well (100 μL/well) and cultured for 3 h or 1 day. After each incubation period, the culture medium was aspirated and 100 μL of DMEM supplemented with 10% FBS was added along with 10 μL of Cell Proliferation Reagent WST-1. For all time points, a standard curve was developed by plating 0.5×10^3, 2.0×10^3, 5.0×10^3, 1.0×10^4, and 1.5×10^4 cells in 100 μL of DMEM serum medium and 10 μL of Cell Proliferation Reagent WST-1. The plates were then incubated for an additional 4 h at 37 °C. After incubation, the absorbance of the samples against the background control on a microtiter plate reader (Bio-Rad Benchmark PlusTM Microplate Spectrophotometer) was obtained at a wavelength of 440 nm with a reference wavelength of 650 nm. Six wells under each condition were used for the measurement to calculate the means and standard deviations.

2.8. RNA isolation and real-time PCR

The MSCs cultured in the wells in the chondrogenic induction medium for 2 weeks were washed with PBS. The cell pellets were taken from the culture plates and frozen in liquid nitrogen. The frozen pellets were crushed into powder by an electric crusher. The powder from each sample was dissolved in 1 mL of Isogen reagent (Nippon Gene, Toyama, Japan) and RNA was isolated. DNase-treated RNA was treated with RQ1 RNase-free DNase (Promega) prior to being converted to cDNA by AMV Reverse Transcription (Takara Bio). Real-time PCR was amplified for GAPDH, types I, II, and X collagen, sox9, and aggrecan. The reaction was performed with 1 μL cDNA, 300 and 150 nM each PCR primer and PCR probe, and TaqMan Universal PCR Master Mix (Applied Biosystems). Reactions were cycled using a Bio-Rad iCycler for 40 cycles. The data were analyzed using Bio-Rad iCycler software. The level of expression of each target gene was normalized to GAPDH. The cell culture and gene expression analysis were performed twice under the same conditions. The primer and probe sequences (Applied Biosystems) followed those of Martin et al. [22] and Schaefer et al. [23]. These sequences were:

GAPDH:(F): 5′-ATGGGGAAGGTGAAGGTCG-3′;
(R): 5′-TAAAAGCAGCCCTGGTGACC-3′;
(probe): 5′-CGCCCAATACGACCAAATCCGTTGAC-3′.
Type I collagen:(F): 5′-CAGCCGCTTCACCTACAGC-3′;
(R): 5′-TTTTGTATTCAATCACTGTCTTGCC-3′;
(probe): 5′-CCGGTGTGACTCGTGCAGCCATC-3′.
Type II collagen:(F): 5′-GGCAATAGCAGGTTCACGTACA-3′;
(R): 5′-CGATAACAGTCTTGCCCCACTT-3′;
(probe): 5′-CCGGTATGTTTCGTGCAGCCATCCT-3′.
Type X collagen :(F): 5′-CAAGGCACCATCTCCAGGAA-3′;
(R): 5′-AAAGGGTATTTGTGGCAGCATATT-3′;
(probe): 5′- TCCCAGCACGCAGAATCCATCTGA -3′.
Sox9 :(F): 5′- CACACAGCTCACTCGACCTTG -3′;
(R): 5′-TTCGGTTATTTTTAGGATCATCTCG-3′;
(probe): 5′-CCCACGAAGGGCGACGATGG-3′.
Aggrecan :(F): 5′- TCGAGGACAGCGAGGCC-3′;
(R): 5′- TCGAGGGTGTAGCGTGTAGAGA-3′;
(probe): 5′-ATGGAACACGATGCCTTTCACCACGA-3′.

2.9. Histological and immunohistological staining

The pellets that formed during cell culture in the chondrogenic differentiation medium were fixed in neutral buffered formalin, embedded in paraffin, and sectioned. The pellet sections were stained with hematoxylin and eosin (H&E) for the nucleus and stained with safranin-O/fast green and toluidine blue to visualize the extracellular GAGs.

The type I collagen, type II collagen, and cartilage proteoglycan were immunohistologically stained using rabbit anti-human type I collagen antibody (Sanbio b.v., Uden, Netherlands), mouse anti-human type II collagen monoclonal antibody (Neomarkers, Fremont, CA), and mouse anti-human cartilage proteoglycan monoclonal antibody that recognizes the short peptides substituted with keratin sulfate side chains and within the core protein of proteoglycans in articular cartilage (Chemicon International, Temecula, CA) and a Dako LSAB Kit, Peroxidase (Dako, Carpinteria, CA) according to the instructions accompanying the kit. Briefly, the deparaffinized sections were incubated with proteinase K enzyme in Tris-buffered saline (1:50 working dilution, pH 7.4, TBS) at RT for 5 min and blocked with peroxidase blocking solution for 10 min and 10% goat serum solution for 30 min. The sections were then incubated with anti-type I collagen (1:400 working dilution), anti-type II collagen (1:200 working dilution), and anti-proteoglycan antibodies (1:1200 working dilution) for 30 min. The biotinylated secondary antibody (anti-rabbit or mouse immunoglobulins) was applied for 30 min followed by incubation with horseradish peroxide-conjugated streptavidin for 10 min. The sections were then incubated with 3-amino-9-ethylcarbazole as the color substrate for 10 min to visualize the bound antibodies. The nuclei were counterstained with hematoxylin. All incubations were at RT.

3. Results

3.1. Surface grafting and characterization

Photoreactive AzPhPAAc was synthesized by coupling PAAc with 4-azidoaniline (Fig. 1a). Photoreactive AzPhPAAm and AzPhPEG were synthesized by coupling PAAm and bis-amino PEG with *N*-(4-azidobenzoyloxy) succinimide, respectively (Fig. 1b–d). The synthesized

AzPhPAAc, AzPhPAAm, and AzPhPEG were purified by dialysis, ultrafiltration, and reprecipitation, respectively. The introduction of the photoreactive azido groups in AzPhPAAm, AzPhPAAc, and AzPhPEG was confirmed by the appearance of two peaks at about 7 ppm in ^{1}H-NMR that were derived from the four protons in the azidophenyl groups. The percentages of the carboxylic groups in the PAAc and the amino groups in the PAAm and bis-amino PEG coupled with the azidophenyl groups were 6.2%, 8.6%, and 100.0%, respectively.

An aqueous solution of AzPhPAAm, AzPhPAAc, or AzPhPEG was eluted in the wells of a 6-well cell culture polystyrene plate and air-dried in the dark. The cast plate was irradiated with ultraviolet light to graft the AzPhPAAm, AzPhPAAc, or AzPhPEG to the polystyrene surface. The contact angles of the polystyrene surfaces modified with PAAc, PAAm, or PEG decreased from 75.7 ± 2.4 degrees to 35.6 ± 3.6, 58.1 ± 4.4, or 53.3 ± 4.3 degrees, respectively. This result indicates that the surface became hydrophilic after surface modification.

The photoreactive polymers were pattern-grafted on polystyrene plates to observe the homogeneity and height of the grafted polymers. The patterning was performed under the same conditions as that of normal surface grafting, but with a photomask. SPM observation of the dried surfaces showed that the photoreactive polymers were homogenously grafted onto the surface. The grafted thicknesses of the dried AzPhPAAm, AzPhPAAc, and AzPhPEG layers were 61.8 ± 6.1, 142.3 ± 6.7, and 59.6 ± 10.2 nm, respectively. The AzPhPEG-grafted surface was rougher than were the AzPhPAAm- and AzPhPAAc-grafted surfaces. Observation of the grafted surfaces under a phase-contrast microscope also showed no evidence of defects of the grafted surfaces. These results indicate that the polystyrene surfaces were homogenously grafted by the photoreactive polymers.

3.2. Cell adhesion, proliferation, differentiation, and biochemical analysis

After culture passage 4, the MSCs were cultured on the modified surfaces and 6-well polystyrene cell culture plates (control) in a chondrogenic induction medium consisting of serum-free DMEM supplemented with dexamethasone and TGF-β3. The cells adhered to the PAAm-modified surface and spread after 30 min culture, spread more after 3 h, and proliferated to confluence after 3 days (Fig. 2). After reaching confluence, the cells gradually aggregated and detached to form pellets. The MSCs on the PAAc-modified and control surfaces were very similar. The cells adhered to the PAAc-modified and control surfaces and spread slightly after 30 min culture. They spread more after 3 h and proliferated to confluence after 3 days. The confluent cells also aggregated and formed pellets, but at a rate slower than those on the PAAm-modified surface. However, the MSCs did not attach to the PEG-modified surface at all and began to aggregate immediately after cell seeding. After 24 h, the cells aggregated into pellets. After 2 weeks, the cells on all surfaces formed pellets. The pellets that formed on the PAAm- and PEG-modified surfaces were larger than were those that formed on the PAAc-modified and control surfaces (Fig. 3).

The DNA and sulfated GAGs contents in each pellet after 2 weeks cultures were analyzed with Hoechst 33258 dye and BlyscanTM GAG assay, respectively. The DNA contents in the pellets formed on the PAAm-, PAAc-, PEG-modified, and control surfaces were 8.71 ± 0.34, 14.82 ± 0.36, 7.08 ± 0.41, 12.42 ± 0.41 μg/mg, respectively. The GAG contents in the pellets formed on the PAAm-, PAAc-, PEG-modified, and control surfaces were 37.71 ± 1.13, 13.98 ± 0.62, 31.43 ± 0.75, 5.60 ± 0.76 μg/mg, respectively. The ratio of GAG to DNA in the pellets formed on the PAAm-, PAAc-, PEG-modified, and control surfaces were 4.33 ± 0.13, 0.94 ± 0.04, 4.44 ± 0.11, 0.45 ± 0.06 μg/μg,

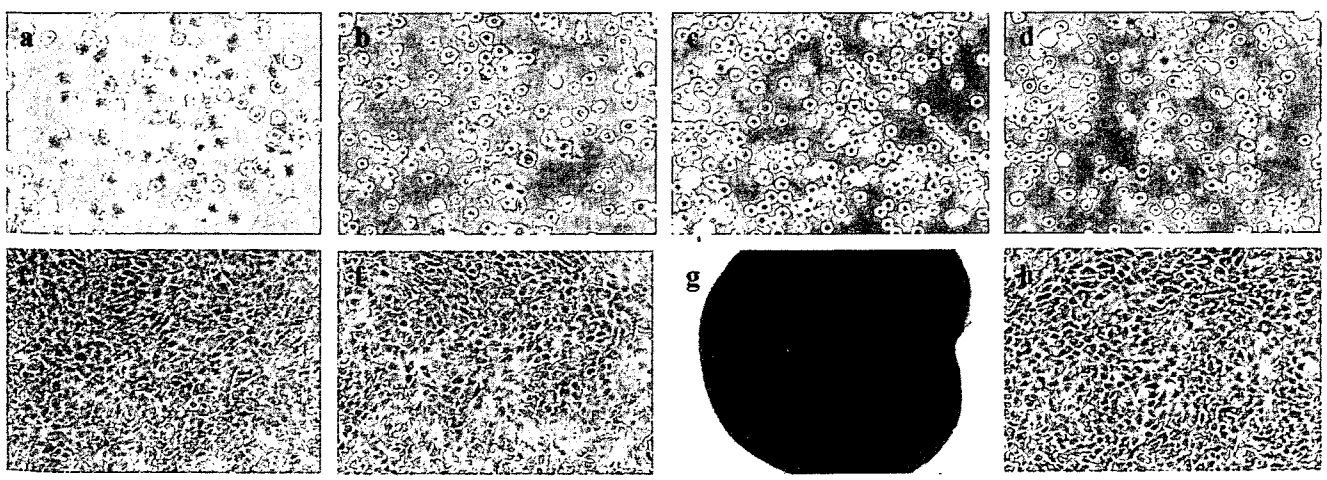

Fig. 2. Phase-contrast micrographs of MSCs cultured on PAAm- (a, e), PAAc- (b, f), PEG-modified (c, g), and control (d, h) surfaces in chondrogenic induction medium for 30 min (a–d) or 3 days (e–h).

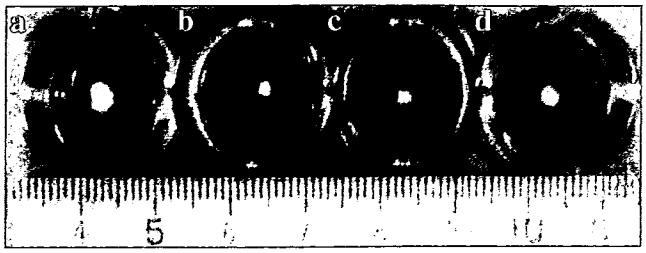

Fig. 3. Pellets that formed on PAAm- (a), PAAc- (b), PEG-modified (d), and control (c) surfaces after cultured in chondrogenic induction medium for 2 weeks.

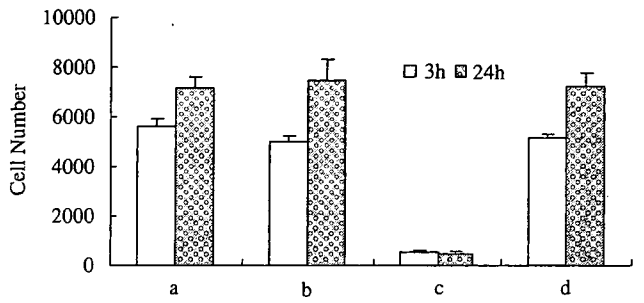

Fig. 4. Proliferation rates of MSCs cultured on PAAm- (a), PAAc- (b), PEG-modified (c), and control (d) surfaces in serum-free DMEM. Data represent the average±SD of six samples.

respectively. The pellets on the PAAm- and PEG-modified surfaces produced significantly more GAG than did those on the PAAc-modified and control surfaces.

The cells proliferated on the PAAm- and PAAc-modified surfaces at almost the same rate as those on the polystyrene cell culture plates (Fig. 4). Almost no cells were detected on the PEG-modified surface because very few cells attached to the surface.

3.3. Histological and immunohistological examinations

The pellets that formed during cell culture were fixed, embedded, and histologically stained with hematoxylin/eosin, safranin O/fast green, and toluidine blue stains (Fig. 5). Histological examination using H&E stains indicated that the cells cultured on the PAAm- and PEG-grafted surfaces had a round morphology; those on the PAAc and control surfaces had a spindle, fibroblast-like morphology. The bright safranin O-positive stain indicated that GAGs were abundant and homogeneously distributed around the cells cultured on the PAAm- and PEG-modified surfaces. Toluidine blue staining revealed the typical metachromasia (purple color) of articular cartilage, coinciding with the results of safranin O staining. However, the cells on the PAAc-modified and control surfaces were not positively stained by safranin O and toluidine blue.

Immunohistological stainings indicate that the pellets that formed on the PAAm- and PEG-grafted surfaces were positively stained with type I collagen, type II collagen, and cartilage proteoglycan, while the pellets that formed on the PAAc-grafted and control surfaces did not show any obvious positive staining for type II collagen and cartilage proteoglycan (Fig. 6). These results indicate that the PAAm- and PEG-grafted surfaces provided microenvironments for MSCs to change to a round morphology and produce cartilaginous ECMs.

3.4. Real-time PCR

The gene expression of type I collagen, type II collagen, type X collagen, sox9, and aggrecan in the pellets was examined by real-time PCR (Fig. 7). The cell culture was performed twice and gene expression of the cells in the pellets from the two cultures was analyzed. The gene expression pattern of the two cultures showed similar trends. After culture passage 4, the MSCs expressed genes encoding type I collagen, a low level of sox9, and aggrecan; they did not express genes encoding type II and type X collagen. The MSCs cultured on the PAAm- and PEG-modified surfaces expressed all these genes. The genes encoding type II and type X collagen, sox9, and aggrecan were upregulated. The cells cultured on the PAAc- and control surfaces expressed genes encoding type I collagen, a low level of sox9, and almost no genes encoding type II and type X collagens and aggrecan. The cartilaginous genes of type II collagen and aggrecan were expressed only by cells cultured on the PAAm- and PEG-modified surfaces. The gene expression results coincided with the histological, immunohistochemical and biochemical results, which indicated that the PAAm- and PEG-modified surfaces promoted the chondrogenic differentiation of the MSCs, but that the PAAc-modified and control surfaces did not.

4. Discussion

Photochemical modification was used to introduce functional groups to cell culture polystyrene plate surfaces. The modified surfaces showed improved water wettability. This method can be used for the surface modification of any organic substrate. The modified surfaces were stable and changed their states in response to the pH of the aqueous solution. Photochemically modified surfaces have been shown to affect cell functions such as cell adhesion, proliferation, and differentiation. The positively charged PAAm-modified surface supported cell adhesion, proliferation, and differentiation. The negatively charged and control surfaces supported cell adhesion and proliferation, but not differentiation. The neutral PEG-modified surface supported neither cell adhesion nor proliferation, but did promote cell differentiation. The PAAm-modified, PAAc-modified, and control surfaces supported MSCs adhesion, but there were some differences among them. The MSCs adhered more rapidly to the PAAm-modified surface than to the PAAc-modified and control surfaces. This difference might be caused by the different electronic properties of these surfaces. The PAAm-modified surface promotes cell adhesion through the electrostatic attractive interaction

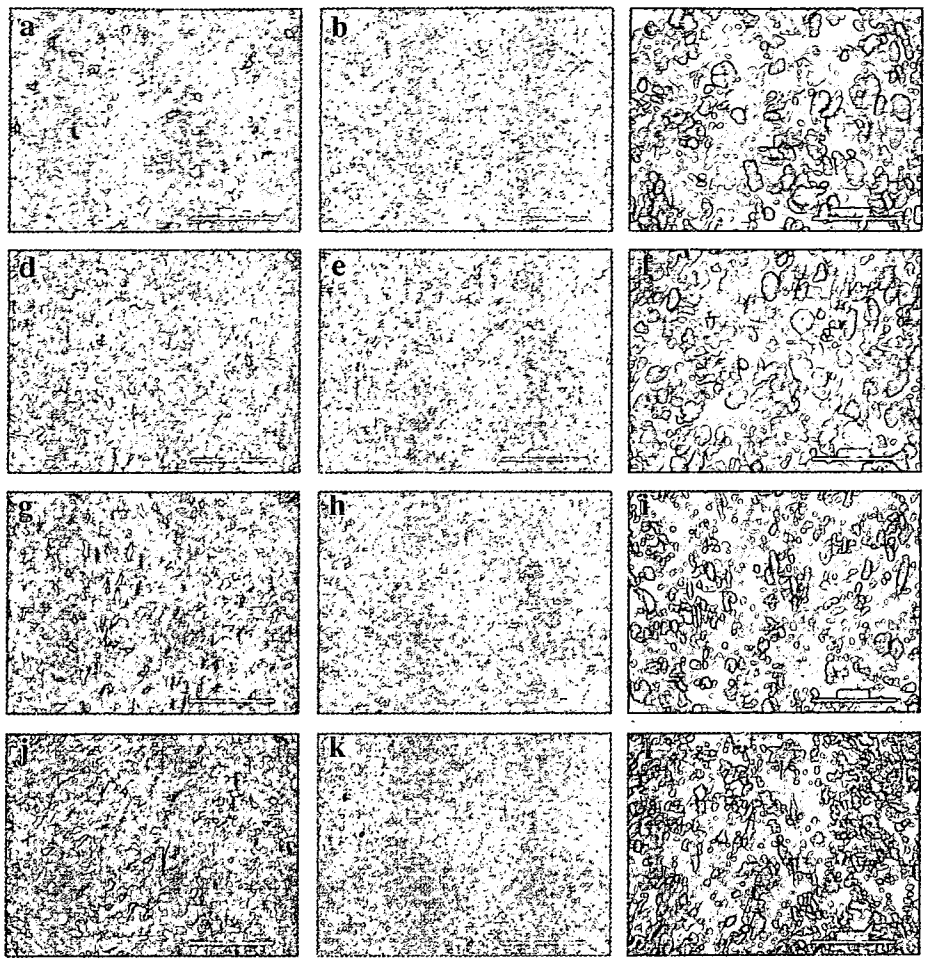

Fig. 5. Hematoxylin/eosin (a, d, g, j), safranin-O/fast green (b, e, h, k), and toluidine blue (c, f, i, l) staining of pellets that formed on PAAm- (a–c), PEG- (d–f), PAAc-modified (g–i), and control (j–l) surfaces after culture in chondrogenic induction medium for 2 weeks. The scale bar is 50 μm.

between the positively charged surface and the negatively charged cells. The negatively charged PAAc-modified and control surfaces do not provide such attractive interaction for cell adhesion. The PEG-modified surface did not have any electrostatic attractive interaction between surface and cells. It also did not support protein adsorption. Therefore, the cells did not adhere to the PEG-modified surface and aggregate directly after cell seeding.

Although the cells cultured on all the surfaces formed pellets, the effects of the PAAm- and PEG-modified surfaces were more evident than were those of the PAAc- and control surfaces. Cells on PEG-modified surface began to aggregate immediately after cell seeding and formed pellets after 24 h. The rapid formation of pellets on the PEG-modified surface indicates that cell–cell interaction is stronger than cell–surface interaction. The cells on the other surfaces also formed pellets after becoming confluent. Shrinkage of the confluent cells may result in the detachment of the cell sheet from the surface and in cell aggregation. The cells in the pellets that formed on the PAAm- and PEG-modified surfaces had a round morphology; expressed cartilaginous genes such as type II collagen, aggrecan, and sox9; were positively stained by safranin O, toluidine blue, anti-type II collagen antibody, and anti-cartilage proteoglycan antibody; and produced more GAG. The PAAm- and PEG-modified surfaces promoted the chondrogenic differentiation of the MSCs. However, the pellets that formed on the PAAc-modified and control surfaces did not display any evidence of chondrogenesis, indicating that these surfaces did not support the chondrogenic differentiation of MSCs. The cells cultured on the PAAm-modified surface in the serum medium also detached and formed pellets, but more slowly than those in the chondrogenic induction medium. However, the cells cultured in the serum medium on the PAAc-modified and control surfaces did not detach and form pellets until 2 weeks culture (data not shown). Culture in the chondrogenic medium facilitated cell detachment from the surface after confluence. A surface grafted with another neutral polymer, poly(vinyl alcohol), showed a similar effect as did that of PEG (data not shown). Although further investigation using other chargeable polymers and longer periods of inductive culture (4 weeks) should be considered, the 2 week culture results using PAAm, PAAc, and PEG clearly

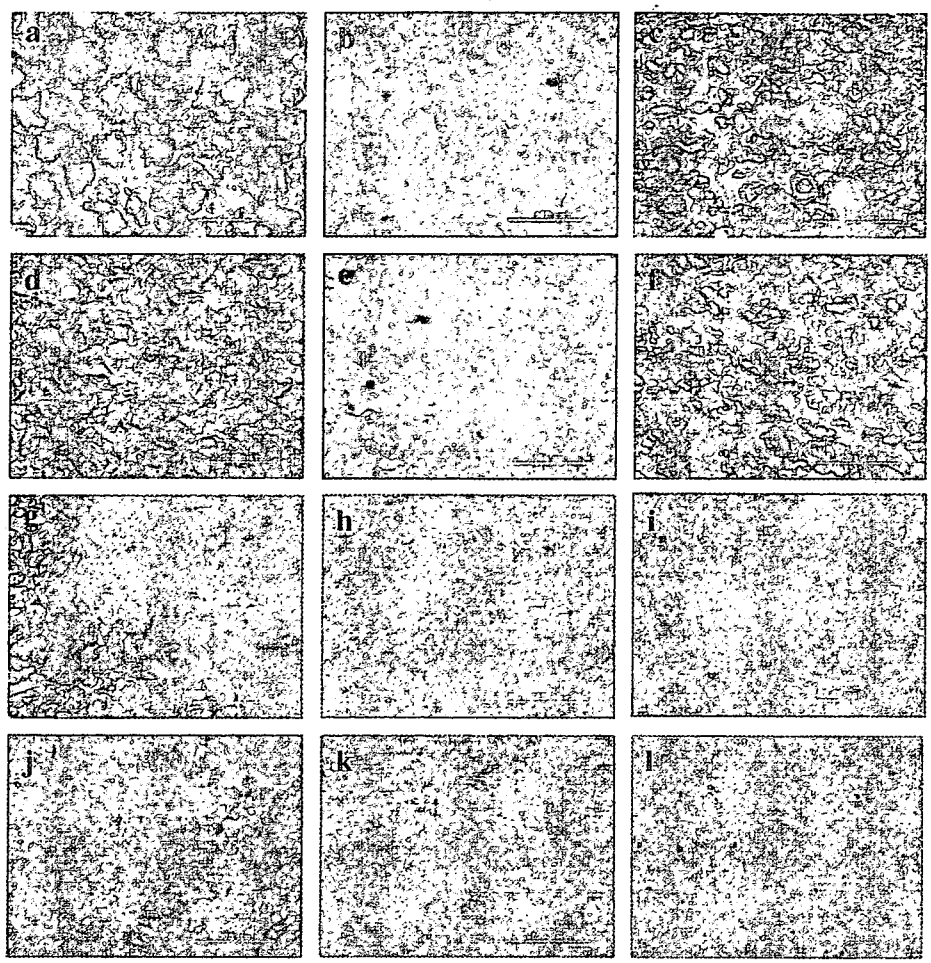

Fig. 6. Type I collagen (a, d, g, j), type II collagen (b, e, h, k), and cartilage proteoglycan (c, f, i, l) staining of pellets that formed on PAAm- (a–c), PEG- (d–f), PAAc-modified (g–i), and control (j–l) surfaces after culture in chondrogenic induction medium for 2 weeks. The scale bar is 50 μm.

demonstrated the early effects of chargeable polymer-modified surfaces on the chondrogenic differentiation of MSCs, and the results might be applicable to other chargeable polymers. Surfaces presenting methyl, hydroxyl, carboxyl, and amino groups have been reported to affect cell functions through modulating fibronectin structure and the availability of binding/receptor sites [24,25]. The different effects of the PAAm-, PEG-, PAAc-modified and control surfaces in the present study may be mediated by adsorbed proteins.

Curran et al. [15,16] reported that $-NH_2$ surfaces did not support chondrogenic differentiation of MSCs in serum medium, but did promote chondrogenesis when cultured in the chondrogenic differentiation medium. –COOH surfaces promote chondrogenesis in both serum medium and induction medium. In the present study, the effect of PAAc-modified surfaces is different from their results. The difference might result from different cell culture conditions. They used a monolayer culture to investigate the effects. However, in this study, at the first stage, the cells on the PAAm-, PAAc-modified, and control surfaces were monolayer and then formed pellets after a few days of culture. The pellets can provide a three-dimensional microenvironment that facilitates chondrogenic differentiation. When cultured on the surface of culture plate for a few passages, primary chondrocytes may change from their original round morphology to a spindle, fibroblast-like shape and lose their ability to express articular cartilage-specific ECMs such as type II collagen and aggrecan. Instead, they express and produce fibroblast-specific ECM, type I collagen [26,27]. The chondrocytes dedifferentiate and change their phenotypes. Three-dimensional microenvironments are necessary to promote cell differentiation [28,29]. Micromass pellet culture is often used in the basic study of the chondrogenic differentiation capacity of MSCs. The formation of pellets on PAAm-, PAAc-, PEG-modified, and control surfaces in the present study should provide additional information regarding the chondrogenic differentiation of MSCs.

5. Conclusions

The electrostatic properties of a biomaterial surface could affect cell functions such as cell adhesion,

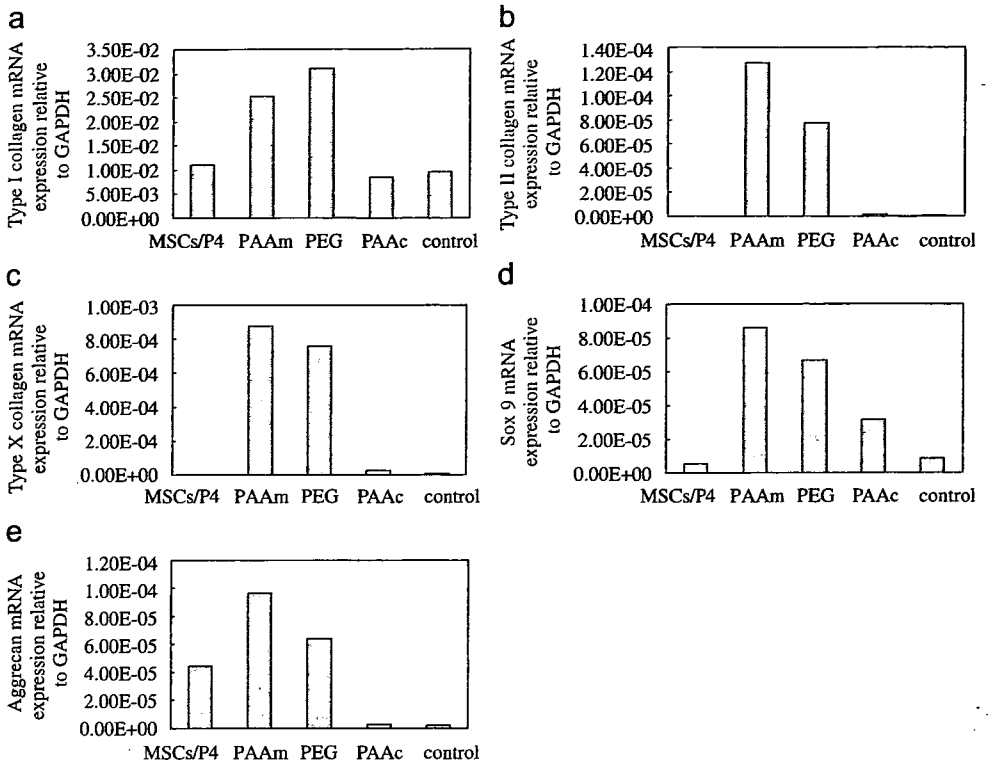

Fig. 7. Real-time PCR results of mRNA expression of type I collagen (a), type II collagen (b), type X collagen (c), sox 9 (d), and aggrecan (e) of the MSCs cultured on PAAm-, PEG-, PAAc-modified and control surfaces in chondrogenic induction medium for 2 weeks. The data are normalized to GAPDH. MSCs/P4 is the cells seeded onto the surfaces.

proliferation, and differentiation. The positively charged PAAm-modified surface supported cell adhesion, proliferation, and the chondrogenic differentiation of MSCs. The negatively charged and control surfaces supported cell adhesion and proliferation, but not chondrogenic differentiation. The neutral PEG-modified surface supported neither cell adhesion nor proliferation, but did promote chondrogenic differentiation. Although both the PAAm- and PEG-modified surfaces promoted chondrogenic differentiation of MSCs, the PAAm-modified surface supported cell adhesion whereas the PEG-modified surface did not. The positively charged PAAm-modified surface is more appealing for tissue engineering because, at first, it can support cell adhesion, and then switch to differentiation of the proliferated cells. Thus, cell proliferation and differentiation could occur on the same surface at different times. Consequently, cell differentiation could be controlled by changing the surface properties such as the electrostatic property. The results of this study will provide important information for the design of scaffolds for tissue engineering.

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Covalently immobilized biosignal molecule materials for tissue engineering

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Immobilization of biosignal molecules including growth factors and cytokines is important for developing biologically active materials which can contribute to tissue engineering as a component. The immobilization has more meanings than only immobilization of the enzyme in a bioreactor or ligand–receptor interactions, because the immobilized biosignal molecules work on cells which have very complex structures and functions. This review discusses recent progress in immobilization of biosignal molecules, including the mechanisms and design concepts.

1. Introduction

Current clinical technologies, especially donor transplants and artificial organs, have been excellent life-saving and life-extending therapies to treat patients who need to reconstitute diseased or devastated organs or tissues as a result of an accident, trauma, and cancer, or congenital structural anomalies. Although advances in surgical techniques, immunosuppression, and postoperative care have improved survival and quality of life, there are still problems associated with the use of biological grafts, such as donor site morbidity, donor scarcity, and tissue rejection. A variety of synthetic and natural materials have been developed for the replacement of lost tissues, but the results have not been satisfactory. Tissue engineering has emerged as a promising alternative in which organs or tissue can be repaired, replaced, or regenerated.[1]

In tissue engineering, a neotissue is generally generated from the cells on a bioresorbable scaffold, incorporating growth factors as shown in Fig. 1. It can consist of up to three components: cells, scaffold, and growth factors. Recent progress in cell biology revealed various types of stem cell. However, because of the scarcity of stem cells it is important to increase the number of stem cells for clinical utilization. Bioreactors to amplify the stem cells are required and, for the construction of bioreactors, some novel materials which enhance the growth or differentiation are desired.[2–6]

For the design of biomaterials, cells and proteins at the interface play an important role.[7,8] Especially for the development of such bioreactor materials or bioactive materials to enhance regeneration in the body, the utilization of biosignal proteins including growth factors and cytokines is reasonable. However, although there are many examples of material design to enhance cell adhesion, there are not so many investigations for regulation of various higher functionalities (gene expression) of cells including growth, differentiation, apoptosis, transformation and so on. In this review the possibilities for regulating the expression of cells by materials are discussed.

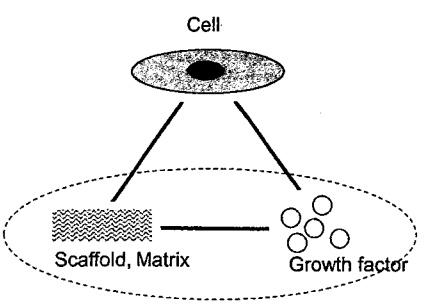

Fig. 1 The principle of tissue engineering. This review focuses on the fusion, that is, immobilization of growth factor on the scaffold.

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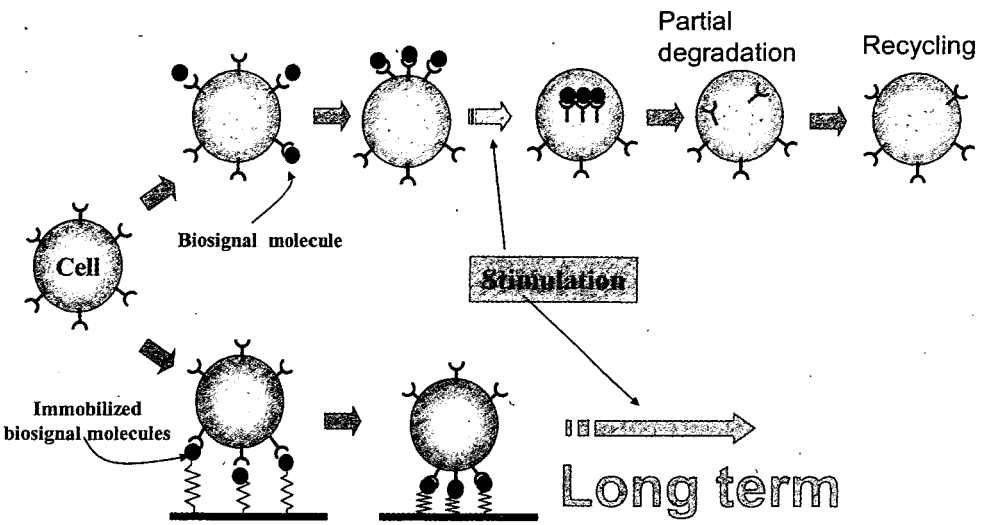

Fig. 2 Interaction of cells with soluble and immobilized growth factors.

2. Immobilization of biosignal molecules

Fig. 2 shows the activation mechanism of biosignal molecules including growth factors and cytokines. First, the biosignal molecules interact with the cognate receptor and form a complex with it, resulting in autophosphorylation of the cytoplasmic domains of the receptors. The phosphorylation activates the intracellular signal transduction. On the other hand, the formed complexes are aggregated and the aggregates are internalized into cells. The internalization occurs by both clathrin-dependent and clathrin-independent mechanisms and leads to the recycling of receptors to the plasma membrane for resensitization or shuttling of receptors to lysosomes for degradatory down-regulation.

If the signal transduction from biosignal molecules to cells is due to complex formation with the receptor, it is expected that the biosignal molecules immobilized on the material surface work. In addition, it is expected that the signal transduction by the immobilized molecules continues for a longer time than by the soluble molecules.

2.1. History of immobilized growth factor

Immobilization of insulin on a solid matrix was for the first time performed and reported by Cuatrecasas.[9] He synthesized the conjugates to investigate the mechanism of insulin action. Although these conjugates facilitated extensive purification of solubilized insulin receptors by affinity chromatography, definitive conclusions regarding the effectiveness of immobilized insulin could not be drawn because of the possibility that the immobilization was incomplete.[10–15] Furthermore, given the marked time delay between the exposure of the cells to the immobilized insulin and the detection of signal transduction events, his study examining acute effects of insulin conjugates may not have allowed sufficient time for evaluation of activity. In addition, because his studies used porous agarose gel beads that were not accessible to the cells as the immobilizing support, it was difficult to evaluate quantitatively the relationship between the amount of immobilized insulin and biological activities.

After these works, in 1991 insulin was immobilized on a plain surface as a growth factor for investigation of its mitogenic (a long-term) effect.[16,17] Subsequently various types of biosignal molecules have been immobilized on materials to regulate cell functions. Table 1 summarizes reports on bioconjugate materials which were covalently immobilized with growth factors to regulate cellular functions.[18–109] Immobilized insulin and epidermal growth factor (EGF) enhanced the growth of cells.[16–65] Bone morphogenetic protein (BMP) −2 or −4 induced alkaline phosphatase activity, or calcium deposition, or regulated gene expression in cells.[73–77] The immobilized transforming growth factor-β1 (TGF-β1) induced collagen synthesis[86,89] or suppressed chondrocytes toward prehypertrophic chrondrocytes and osteolineage cells.[88] Notch ligands induced Notch activation in cells only in the immobilized state and it was suggested to expand human hematopoietic stem cell population.[93–95] Immobilization of leukemia inhibitory factor (LIF) kept the colony morphology, alkaline phosphatase activity, and stage-specific embryonic antigen-1 immunoreactivity of embryonic stem cells, which indicated an undifferentiated state.[96,97]

On the other hand, in around 1990, in the field of cell biology, biologically natural "juxtacrine stimulation", which indicates biological signal transduction in a nondiffusible manner to neighboring cells, was demonstrated for some membrane-anchored growth factors and lymphokines, including TGF-α, tumor necrosis factor α, colony-stimulating factor 1, c-kit ligand, and heparin-binding EGF.[110,111] Considering the biological system, Ito *et al.* named the effect induced by a chemically immobilized growth factor "artificial juxtacrine stimulation".[112] Recently, some reviews for biomimetic approaches to biomaterials, which signal to cells *via* biologically active entities, have been published.[7,113]

2.3. Interaction of immobilized growth factor

In order to demonstrate that the immobilized signal molecules work to transduce the signal to the cells, some works have been performed. Chen *et al.*[27] prepared three basic types of insulin

Table 1 Biosignal molecules covalently immobilized on a matrix for cell culture

Growth factor	Substrate	Reference
Insulin	Surface-hydrolyzed poly(methyl methacrylate) (PMMA)	16–21
	Surface-treated glass or polyacrylamide	22
	Polyurethane	23
	Biodegradable polymer	24
	Poly(2-hydroxyethyl methacrylate) (poly(HEMA))	25
	Spacer, surface-treated PMMA with POE	26
	Polymer grafted with poly(acrylic acid)	27, 28
	Polyurethane with POE	29
	Biodegradable polymer + POE	30–32
	+ Adhesion factors (RGDS (Arg-Gly-Asp-Ser), pollyallylamine, collagen, *etc.*)	33–40
	+ Heparin	41
	Polystyrene + micropatterning	42, 43
	Poly(*N*-isopropylacrylamide)	44
	Poly(*N*-isopropylacrylamide) + RGDS	45–47
Epidermal growth factor (EGF)	Surface-modified glass	48–50
	Surface-hydrolyzed PMMA	51
	Surface-modifed PDMS	52, 53
	Polystyrene photo-immobilized (mircopatterned)	54–59
	Gene-engineered	60–66
Nerve growth factor (NGF)	Surface-modified glass	49, 67
	Poly(HEMA) grafted with PAA	68, 69
	Gelatin tricalcium phosphate crosslinked	70
	Micropatterned	71, 72
Bone morphogen protein (BMP)	Surface-modified titanium	73, 74
	Type I atelocollagen	75, 76
	Chitosan nanofiber	77
	Poly(lactide-*co*-glycolide)	78
Vasular endothelial growth factor (VEGF)	PAA-grafted polyethylene film	79
	Micropatterned	80
	Gene-engineered	81
Fibroblast growth factor (FGF)	Photoimmobilization	82
	Polymers	83
	+ Heparin	84, 85
Insulin growth factor-1 (IGF-1)	Photoimmobilization	82
Transforming growth factor-β1 (TGF-β1)	Collagen type I coated titanium	86
	PDMS	87
	Gelatin-hyaluronic acid-chondroitin-6-sulfate sponge	88
	+ POE	89
Hapatocyte growth factor (HGF)	Gene-engineered	90, 91
Notch ligand	Photo-immobilized	92
	Gene-engineered	93–95
Leukemia inhibitory factor (LIF)	Photo-immobilized	96
	Non-woven polyester fabrics	97
Stem cell growth factor (SCF)	Gene-engineered	98
Interleukin-2	Crosslinked	99
Interleukin-1	Gene-engineered	100
Tumor necrosis factor-α (TNF-α)	Photo-immoblized	67,101
Erythropoetin	Photo-immobilized with gelatin	102
Neurotrophin-3	Surface-modified glass	50
Transferrin	Surface-hydrolyzed PMMA	103
E-cadherin	Gene-engineered	104

Table 1 Biosignal molecules covalently immobilized on a matrix for cell culture (*Continued*)

Growth factor	Substrate	Reference
Osteopontin	Poly(2-hydroxyethyl methacrylate)	105
	Collagen	106
P-selectin	Coated on polystyrene	107
CXCR3 ligand	Coated on polystyrene	108
Sonic hedgehog	Gene-engineered + interpenetrating polymer network	109

conjugate and their effect on cell growth was investigated. The insulin–polyoxyethylene conjugate (Ins–POE), insulin–poly(acrylic acid) conjugate (Ins–PAA), and insulin–immobilized PAA-grafted polystyrene (Ins–PSt). Ins–POE and Ins–PAA were monovalent and multivalent water-soluble conjugates, respectively. On the other hand, Ins–PSt was a water-insoluble multivalent conjugate. The mitogenic activity of Ins–POE was lower than that of native insulin. This could be due to the inability of the insulin receptor to bind to the coupled insulin because of steric hindrance from the polyoxyethylene chain. With similar reasoning, poly(acrylic acid) should inhibit the binding of insulin to its receptor. However, Ins–PAA showed a slightly higher mitogenic activity compared to native insulin, presumably because Ins–PAA was multivalent and hence able to enhance receptor dimerization as well as aggregation of the insulin conjugate/receptor complex. On the other hand, Ins–PSt had an extremely high mitogenic effect being much higher than the other conjugates. Similar results were obtained using different types of matrix[28] and using the immobilized EGF[51] as summarized in Fig. 3.

2.4. Certification of the interactions

As discussed previously,[10–15] in the case of immobilization of biosignal molecules on a solid, the completeness is very important. If there are some releases from the matrix, the effect may be due to some artifact. Therefore the amount of immobilized insulin was carefully measured by radioisotope labeling.[20] In addition, the immobilized growth factor was repeatedly used after each cell culture.[21–102] This repeated use confirms the stability of the immobilized molecules. In addition to these methods, there are some technologies to demonstrate the activity of immobilized biosignal molecules.[14]

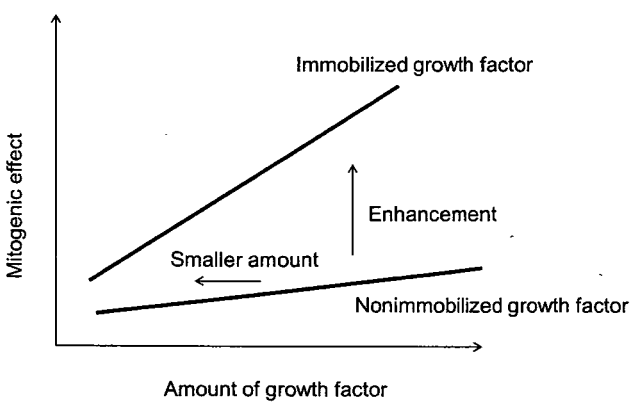

Fig. 3 Schematic comparison of the mitogenic effect of immobilized growth factor with that of nonimmobilized growth factor. Immobilized growth factor induces a greater effect than nonimmobilized growth factor. The smaller amount of immobilized growth factor is considered to induce the effect as a result of multivalency and high local concentration, as discussed in sections 3.1 and 3.3, respectively. The enhancement of the mitogenic effect is considered to be caused by inhibition of down-regulation and other factors as discussed in sections 3.2 and 3.3, respectively.

Specific interaction of immobilized growth factor with the cognate receptor was examined by antibodys against immobilized growth factor. If the effect of the immobilized growth factor was inhibited by the antibody, the specific interactions were certified. This method has been employed by many researchers.[14,19,80,110,111]

One of the technologies to certify the interactions, visualization of the signal transduction and spatial regulation of the cell functions, was performed by micropattern-immobilization using a photo-lithographic method.[42,43,54–59,67,71,72,80,92,102] When the cell culture was on the micropattern-immobilized growth factor, the cell growth or differentiation was accelerated only in the biosignal molecule-immobilized areas. The immobilized biosignal molecules affected the cell functions including growth and differentiations without enhancing the adhesion.

To further investigate the interaction of immobilized EGF, CHO cells overexpressing EGF receptors were cultured on the plate immobilized with EGF in a narrow stripe pattern.[55] The contact area (stripes 2 μm in width) between the cells and the immobilized EGF was stained by an anti-phosphotyrosine antibody. Since free lateral diffusion and internalization of the bound EGF–EGF receptor complex were prohibited by immobilization of EGF, only signal proteins in the interaction regions were activated. This finding also indicates that the biological signal was transduced only to the cell that interacted with the immobilized EGF.

Recently Ichinose *et al.*[49] quantitatively evaluated the interaction. When the density of EGF was only slightly lower than that of the EGF receptor dimers, cellular response was dramatically decreased. The EGF receptor molecules bound with the immobilized EGF were prevented from being laterally diffused and internalized and kept their initial position. In addition, the immobilization made suitable targets for stable single molecule observation under total internal reflection fluorescence microscopy to study EGF signaling mechanisms, preventing lateral diffusion and internalization of EGF receptors. Ichinose *et al.* showed results of single molecule observations of the association and dissociation between phosphorylated EGF receptors and Cy3-labeled growth factor

receptor-binding protein 2 (Grb2) proteins in A431 cells stimulated by the immobilized EGF.

Shibata *et al.*[67] demonstrated that nerve growth factor (NGF)–receptor complexes had two distinct diffusive states, characterized as a mobile and an immobile phase. The transition between the two diffusive states occurred reversibly with duration times determined by a single rate limiting process. The abrupt transition to the immobile phase often occurred simultaneously with the clustering of the NGF–receptor complexes. Immobilization depended on the phosphorylation of the TrkA NGF–receptor. Using dual-color imaging, it was demonstrated that the membrane recruitment of the intercellular signaling protein occurs with NGF–receptor complexes in the immobile phase indicating that signal transduction occurs during this phase. Thus, it was considered that NGF signaling was performed through a repetitive random process to induce formation of signaling complexes.

Previously the effect of immobilized growth factor was compared with the effect of adhesion factors including gelatin, collagen, and fibronection, or with albumin, immunoglobulin.[18] Recently microarray systems have been employed to investigate the effect of immobilized molecules by some researchers.[50,82,114,115] The recently developed systems also revealed the effectiveness of immobilized growth factors. At least the result that the immobilized biosignal molecules stimulate the cells has been confirmed by many methods.

3. Effect accompanying immobilization of growth factors

In addition to the fact that the immobilized growth factors interact with the cellular cognate receptors, it has been demonstrated that the immobilized growth factor had a higher or different effect from the soluble one as shown in Fig. 3, because the interaction is not only the ligand–receptor interaction. These effects of immobilized growth factor are considered to be due to the following mechanisms.[21]

3.1. Multivalency

The importance of mutivalency of immobilized biosignal molecules have been discussed by many researchers. This effect is the same as the high local concentration of immobilized proteins on material surfaces.[16,116] Recently Kiessling *et al.* considered synthetic multivalent ligands as probes of signal transduction.[117] Multivalent ligands can bind avidly to multiple receptors on the cell surface, a process that is facilitated in the fluid lipid bilayer by the two dimensional diffusion of receptors. The multivalent ligands can activate signaling pathways if they can cluster signaling receptors (Fig. 4). They discussed the three major concepts that are critical for the application of multivalent ligands as probes of signal transduction: (1) signal transduction cascades are mediated by receptor–receptor interactions, and promoting receptor assembly is critical for signaling. (2) Multivalent ligands can interact with the target receptors through multiple binding modes. (3) The structure of a multivalent ligand will determine the favored binding modes. Thus, the structure can be optimized to elicit the desired biological response.

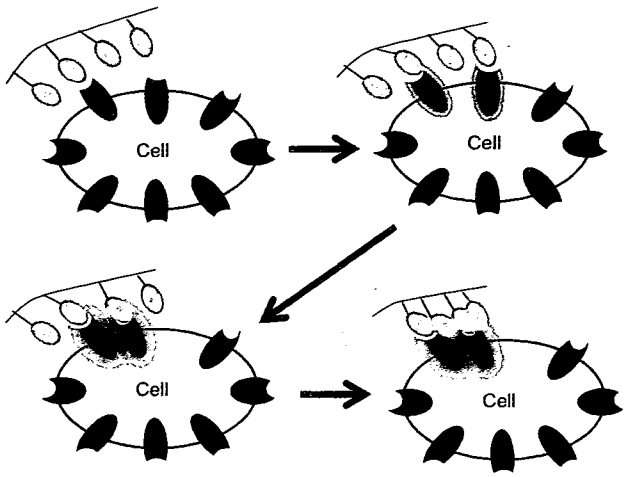

Fig. 4 Interactions of a multivalent ligand with a cell. The multivalent ligand enhances the formation of ligand–receptor complexes, and the interaction of activated ligand–receptor complexes, because of the high local concentration of ligands. In addition, the formed complexes are stabilized by the multivalent ligands.

In addition, prevention of lateral diffusion of the activated receptor or the receptor–ligand complex in the plane of the cell membrane must be taken into consideration. The prevention means the stabilization of the aggregates of complexes and thus leads to a long-lasting effect.

3.2. Inhibition of down-regulation

Inhibition of the internalization process should be also taken into consideration as shown in Fig. 2. Generally cells decompose the biosignal molecules in the cells to reduce their stimulation and this is called down-regulation. The immobilization was considered to inhibit this down-regulation and as a result the stimulation continued for a long time without reduction.

To investigate the above hypothesis, the activation of cellular signaling proteins, insulin receptor β-subunits in the cells, or mitogen-activated protein kinase (MAPK) in the cells was measured in the presence of immobilized insulin or immobilized EGF (Fig. 5).[21,51] Native insulin or EGF rapidly activated the insulin or EGF receptor. However, the activation is usually transient. On the other hand, activation by the immobilized insulin or EGF continued to increase up to 12 h although some lag time was needed for adhesion of cells on the surface immobilized with insulin or EGF. These sustained activations of signaling proteins by the immobilized insulin or EGF should explain the high mitogenic effect of the immobilized growth factors.

In addition, this sustained activation provided another effect of immobilized biosignal molecules.[58] It is well known that growth of the rat phaeochromaocytoma cell line PC12 is stimulated by EGF and the differentiation is stimulated by NGF.[118] However, the immobilized EGF stimulated PC12 differentiation.[58] The immobilized EGF caused a long-lasting stimulation of MAPK and a subfamily of the MAPK superfamily in cells, as did diffusible NGF. This switching between growth stimulation and differentiation was considered to be due to the duration of stimulus.[58]

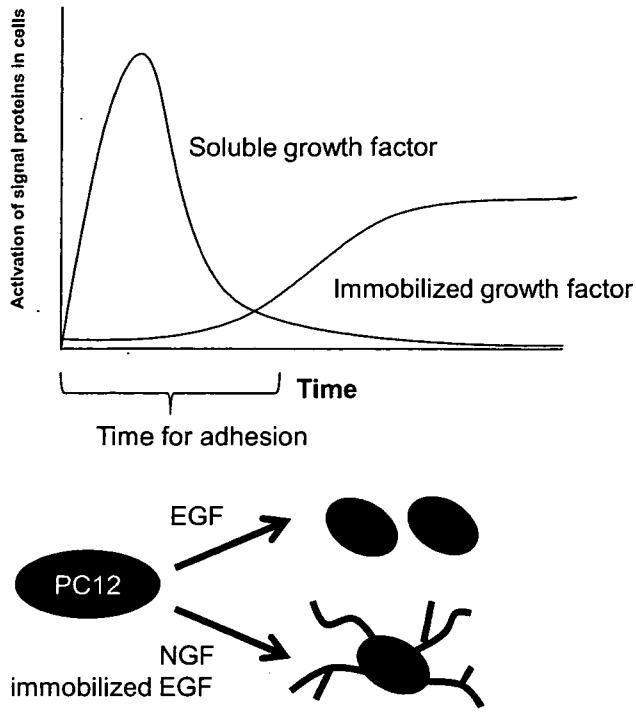

Fig. 5 Time course of signal activation by immobilized and soluble growth factors. The difference induces different effects on PC12 cells.

3.3. Other considerable mechanisms

In addition, the difference in stimulation sites between the cell–medium interface and cell–matrix interface, *etc.* should also be taken into consideration. The interaction of immobilized biosignal molecules is from the substrate and the local concentration is extremely high as shown in Fig. 6. The specific situation is considered to affect various functions.

In fact, the immobilization sometimes induced some specific effect on cell behavior in addition to the primary effect. Kuhl and Griffith-Cima[48] found that the rounding responses of primary rat hepatocytes on the surface immobilized with EGF were different to those on the non-immobilized. Ogiwara *et al.*[63] reported that the cytoskeleton of A431 cells adhering to immobilized EGF-Fc (Fc is a fragment of immunoglobulin) was filopodia whereas that of the cells adhering onto collagen in the presence of soluble EGF was lammellipodia. In addition, Ogiwara *et al.*[64] found that interaction between photo-immobilized EGF and the receptor in the cells was independent of Mg^{2+} although integrin-mediated cell adhesion to natural exracellular matrices is dependent on Mg^{2+}. Phosphorylation of EGF receptors in A431 cells was induced by immobilized EGF the same as soluble EGF. DNA uptake of hepatocytes decreased with immobilized EGF whereas it increased with soluble EGF. Liver-specific functions of hepatocytes were maintained for 3 d by immobilized EGF whereas they were not maintained by soluble EGF, indicating that immobilized EGF follows a different signal transduction pathway from soluble EGF. These differences with immobilized growth factors compared to the soluble ones should be due to the complex structure of interfaces surrounding the cultured cells.

Reddy *et al.*[119] demonstrated an approach derived from understanding how the attenuation mechanisms including growth factor depletion and receptor down-regulation arise from ligand/receptor trafficking processes. A recombinant EGF mutant with reduced receptor binding affinity is a more potent mitogenic stimulus for fibroblasts than natural EGF because of its altered trafficking properties. Optimization of ligand binding parameters requires systemic integration of processes from the initial binding event to the final cellular response. Their experimental data showed that consideration of cellular trafficking processes is essential for an optimization effort. The immobilized growth factor may have a similar effect.

4. Design of immobilization

In order to efficiently derive the effect of immobilized biosignal molecules for material design, there are many strategies reported (Fig. 7).[120]

4.1. Spacer insertion and surface stiffness

As shown in Fig. 2, after the ligand complexes with the receptor, the complexes are generally considered to aggregate in the cell membrane to transmit the signal to the nuclei.

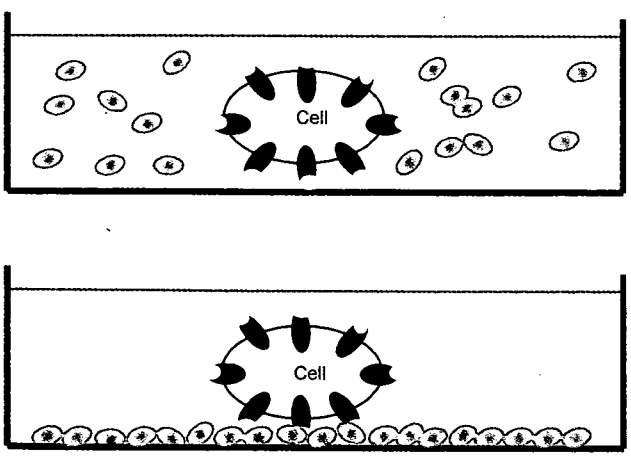

Fig. 6 Immobilized growth factor, which is different from soluble growth factor, provides a different environment for cells.

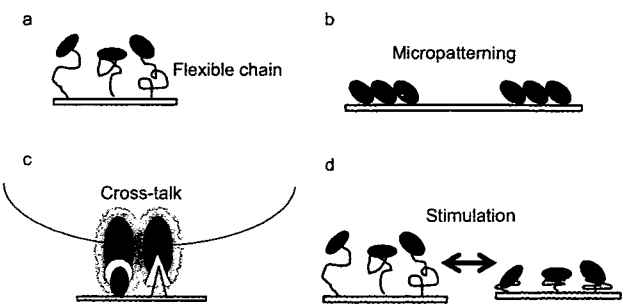

Fig. 7 Material designs using immobilized growth factors. (a) Flexible spacer chains make the immobilized growth factor mobile like a soluble growth factor. (b) Micropattern-immobilization for growth factors induces micropattern formation of cells. (c) Co-immobilization of different types of ligands (growth factor) induced cross-talk of receptors. (d) Stimuli-responsive polymers add some functions to the growth factor immobilization.

Therefore, the insertion of the spacer between the surface and the biosignal molecules is useful for enhancement of diffusion of the complex in the membrane. Some studies using the spacer chains were performed and the increase in the activity is reported.[26,29–32,48] However, possible implications of steric hindrance in spacing growth factor from the substrate and the possible impact on growth factor binding to its cell surface receptor should be taken into consideration. In fact, the POE conjugation reduced the biological activity of insulin.[27]

The insertion of a spacer arm is also related to the surface stiffness. It is well known that the cells sense and react to extracellular stiffness as revealed by recent experiments with soft elastic substrates.[121,122] Therefore, immobilization was performed on polymeric materials having different water contents and the effect of immobilized growth factor was discussed. The increase in flexibility of immobilized growth factor increased the biological activity.

Iwamoto and Mekada[111] discussed the juxtacrine mechanism according to the flexibility of membrane-anchored growth factor, heparin-binding epidermal growth factor (HB-EGF). Stable transfectants of mouse L cells expressing precursor HB-EGF (proHB-EGF) were fixed with formalin to prevent the release of soluble HB-EGF (sHB-EGF), and then the fixed donor cells were cultured with EP170.7 cells, an EGF receptor–ligand dependent cell line. Under these conditions, growth stimulation of EP170.7 cells was observed and this EP170.7 cell growth was found to be dependent on the amount of proHB-EGF expressed on the donor cells. On the other hand, in order to examine the activities of proHB-EGF, a modified coculture system was reported, in which an intact monkey kidney cell line expressing proHB-EGF was incubated with EGF receptor-expressing 32D cells. Under these conditions, proHB-EGF was shown to have a growth-inhibitory activity and to induce apoptosis of the recipient cells, while sHB-EGF stimulated cell growth. They consider that the fixation by formalin perturbed the aggregation of ligand/receptor complexes in the cell membrane.

4.2. Micropatterning

Biomaterials for cell patterning have been used to regulate cellular processes, such as proliferation and differentiation, through cellular adhesion.[120,123–132] As cells adhere to micropatterned substrates, they align themselves to the shape of the underlying adhesion region. This shape change induces changes in cytoskeleton features and has been shown to influence apoptosis and proliferation.[133] Gene expression and protein synthesis were also altered by changing the nuclear shape.[134] It has been determined that cell shape can also control stem cell differentiation[135] and that the tissue form itself can feed back to regulate patterns of proliferation through micromechanical forces.[136] Controlling the cellular microenvironment through micropatterning may be used for directing cell fate for tissue engineering applications.

Moreover, inside the body, cells lie in contact or in close proximity to other cell types in a tightly controlled architecture.[125] Tissue engineering constructs, which aim to reproduce the architecture and geometry of tissues, will benefit from methods of controlling cell–cell interactions within these tissues. Patterned cocultures are a useful tool for tissue engineering constructs and for studying cell–cell interactions *in vitro* because they can be used to control the degree of homotypic and heterotypic cell–cell contact. Pioneering work in this area was performed by studying the interaction of hepatocytes and nonparenchymal fibroblasts in cocultures.[137] Recently developed methods are based on thermally reversible polymers,[138] layer-by-layer deposition of ionic polymers,[139] microfluidic deposition,[140] and molding of hydrogels.[141]

As mentioned in section 3.1, the micropatterning is also useful for investigating the effect of immobilized growth factors. If no signal transduction is observed on non-immobilized growth factor, it is possible to conclude that there is no release of immobilized growth factor to act on the cells.[112,120]

In addition to the certification method, micropatterning has been employed for regulation of cellular morphology and tissue formation. Ito *et al.*[80] demonstrate the micropatterning of blood endothelial cells to form vessels. Gomez *et al.*[72] reported axon extension in neurons on the surface covalently immobilized with NGF and microtopography was introduced in the form of microchannels. When the two surface stimuli were presented in combination, a synergistic increase in axon length was detected, which could be a result of faster polarization triggered by topography plus enhanced growth from NGF.

4.3. Co-immobilization

In order to enhance the effect of immobilized growth factors, other macromolecules have been co-immobilized with them. Biological or physico-chemical enhancement of cell adhesion increased the mitogenic effect of immobilized growth factors.[33–40,45,46] For biological enhancement of cell adhesion, cell adhesion factors including collagen, fibronectin, gelatin, and the core RGDS peptide were co-immobilized. On the co-immobilized surface both adhesion and growth of cells were remarkably enhanced. For physico-chemical enhancement of cell adhesion, cationic polymers such as poly(allyl amine) and polylysine were employed.

In addition, recently various types of stimuli-responsive materials were developed and cells were manipulated on these materials.[142–145] Chen *et al.*[44] immobilized insulin with a thermo-responsive polymer, poly(*N*-isopropylacryamide). They observed growth enhancement by immobilized insulin and harvested the cells by lowering the temperature. Coimmobilization with insulin and RGDS peptide was reported by Hatakeyama *et al.*[45–47] recently.

4.4. Protein engineering for immobilization

Recently many types of gene-engineered proteins for immobilization have been reported. Nishi *et al.*[60] constructed fusion proteins consisting of a growth factor moiety and the collagen-binding domain (CBD) of collagenase, which acted as an anchor to the collagen fibrils. They chose EGF and basic fibroblast growth factor (bFGF) as parts of the fusion proteins (collagen-binding EGF, CBEGF; collagen-binding bFGF, CB-FGF). As a result, CBEGF, when injected subcutaneously into nude mice, remained at the sites of injection for up to 10 d, whereas EGF was not detectable 24 h after injection. Although

CBEGF did not exert a growth-promoting effect *in vivo*, CBFGF, but not bFGF, strongly stimulated the DNA synthesis in stromal cells at 5 d and 7 d after injection. These results indicate that CBD may be used as an anchoring unit to produce fusion proteins nondiffusible and long-lasting *in vivo*.

Hayashi *et al.*[61] developed a recombinant technology to confer mitogenic activity on type III collagen by fusing it to EGF at the collagen's N-terminus. The fusion protein was shown to hold the triple helical conformation of collagen and the mitogenic activity of EGF. It was also demonstrated that the chimeric protein can be immobilized on tissue culture dishes as a fibrous form and in collagen fibrils without abolishing the original mitogenic activity of EGF.

Collagen-binding or fibrin-binding growth factors consisting of EGF, HGF, and VEGF and the binding domains in fibronectin were reported as shown in Fig. 8.[62,90] The fusion protein bound to gelatin and fibrillar collagen sponges and substantially stimulated cell growth after binding to collagen-coated culture plates, whereas EGF or HGF had no effect, indicating that this fusion protein acted as a collagen-associated growth factor. On the other hand, Elloumi *et al.*[65] reported a novel protein consisting of a RGD (Arg-Gly-Asp) sequence functioning as a cell adhesive function, EGF as a cell growth function, and a hydrophobic sequence as an efficient assembling function in one molecule. The protein coated on an unmodified hydrophobic surface of a cell culture plate (through the hydrophobic moiety) retained both cell adhesive activity (through the RGD sequence) and cell growth activity (through the EGF moiety).

Recently Kim *et al.*[100] prepared a fusion protein of a recombinant human interleukin-1 (IL-1) receptor antagonist and an elastin-like peptide (IL-1ra-ELP) and found that the immobilized IL-1ra-ELP modulates the inflammatory profile of lipopolysaccharide (LPS)-stimulated cultured human monocytes. Specifically LPS-stimulated THP-1 monocytes that were exposed to either soluble or immobilized IL-1ra-ELP did not differentiate, but showed attenuated expression of pro-inflammatory cytokines, and had enhanced production of anti-inflammatory and pro wound-healing cytokines. The extent of signaling by immobilized and soluble fusion proteins were similar in magnitude, indicating roughly equivalent bioactivity and that cultured monocytes are clearly signaled by the immobilized IL-1ra-ELP.

As another binding method, fusion with immunoglobulin G Fc region,[63] *p*-azido phenylalanine,[64] histidine tag,[66] cysteine-containing tag[81] and cellulose-binding domain[98] were employed. These gene engineering methods contributed oriented immobilization of biosignal molecules for efficient interaction with receptors to induce cellular signal transduction.

5. *In vivo* applications

Covalent immobilization of growth factor onto a substrate has been used mainly for cell culture. However, some *in vivo* experiments have also been performed for tissue engineering. Ohyama *et al.*[85] prepared bFGF-immobilized platinum microcoils and performed coil embolization of aneurysms constructed using a canine common carotid artery *via* the endovascular approach. The percentage of occlusion at the aneurysm orifice in animals treated with bFGF-immobilized coils was significantly greater than with unmodified coils. Liu *et al.*[78] immobilized BMP-2 on a poly(lactide-co-glycolide) scaffold and created bilateral, full-thickness cranial defects in rabbits to investigate the osteogenic effect of cultured mesenchymal stromal cells on bone regeneration *in vivo*. Histomorphometry and histology demonstrated that the BMP-2 conjugate enhanced bone formation after surgery. Nishi *et al.*[60] prepared collagen-binding EGF and injected it subcutaneously into nude mice. It remained at the sites of injection for up to 10 d, whereas EGF was not detectable 24 h after injection. In addition, collagen-binding FGF strongly stimulated the DNA synthesis in stromal cells at five and seven days after injection. Kitajima *et al.*[90] prepared collagen-binding HGF and its angiogenic activity in rat tissues was examined by subcutaneously implanting collagen sponges containing the binding HGF. Blood vessel formation in the sponges after seven days was four to six times more extensive when compared with the control sponges without any sample. Implanted sponges with native HGF did not show significant any difference from the controls. Recently, Ohkawara *et al.*[91] studied the re-endothelialization and neointimal formation in balloon-injured rat carotid arteries in the presence of HGF and binding HGF. The left common carotid artery of male Sprague Dawley rats was injured with an inflated balloon catheter, and treated with binding HGF, native HGF, or saline (control) for 15 min. Rats were injected with Evans blue and

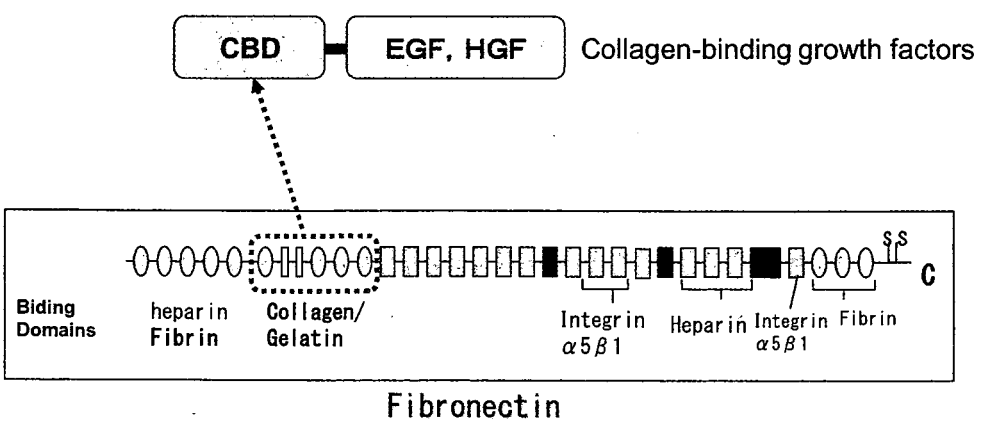

Fig. 8 Gene-engineered growth factors (EGF, VEGF, HGF, *etc.*) containing collagen-binding domain (CBD) found in fibronectin.