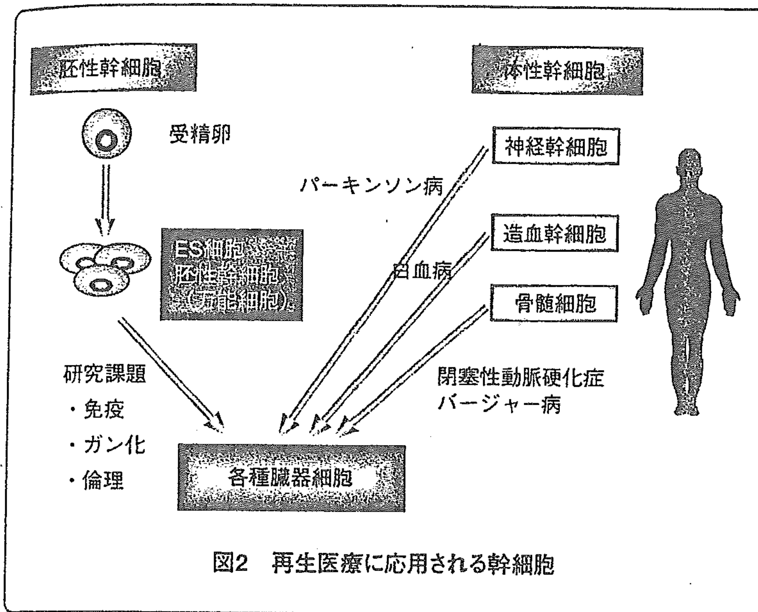


図2　再生医療に応用される幹細胞

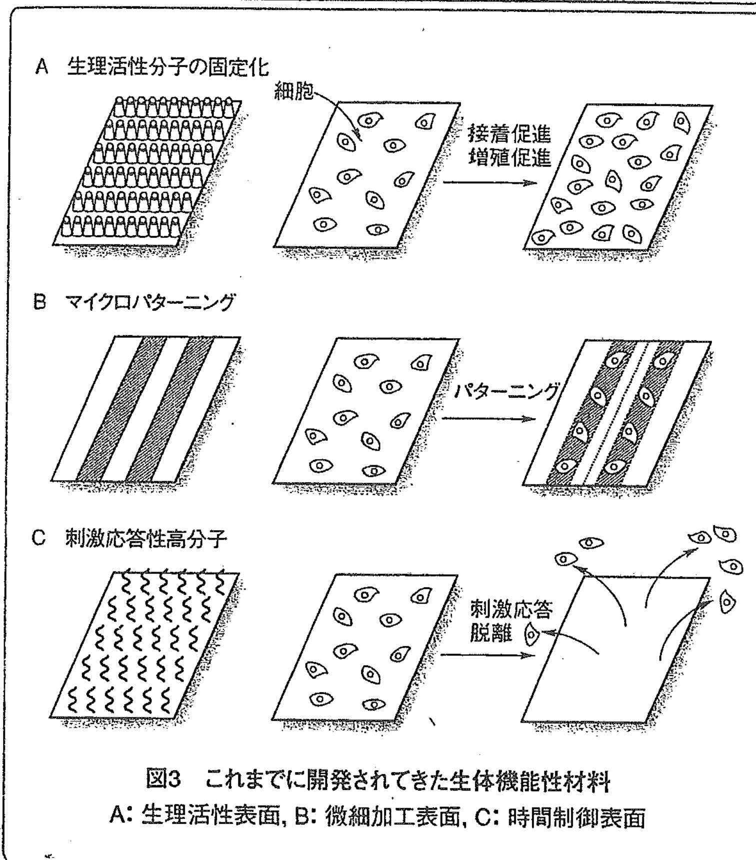


図3　これまでに開発されてきた生体機能性材料
A: 生理活性表面, B: 微細加工表面, C: 時間制御表面

グが提唱されるようになりました。そして20世紀が終わろうとする頃に，上述の医学上の2大発見により再生医療が生まれてきたのです。

1. 再生医療

再生医療は，「幹」細胞技術を医療に応用しようとするものです。幹細胞とは，多分化能をもち自己複製可能な細胞です。**図2**に示すように，再生医療で重要となる幹細胞は二つに分類することができます。ES細胞については，前述のようにまさに応用に向けた研究が始まったところです。これに対して，最近，成人の体の中にも体性幹細胞が存在することが報告されるようになり，再生医療のブームが世界に広がりました。体性幹細胞として，古くから知られているものには造血幹細胞があり，これは既に多くの骨髄，末梢血，臍帯血移植が行われ，患者の造血系を再生できることがわかっています。また，患者自身の骨髄を使った壊死組織の再生治療は03年から高度先進医療に認定され，幹細胞を本来ある場所と異なるところに移植して治療することも臨床で行われるようになっています。

このように着々と進歩する再生医療ですが，さらに発展させるためには今後解決していかなければならない課題がいくつもあります。その中で，材料の新しい性能を利用した再生医療技術が期待されています。

2. 細胞機能制御基材の基礎

まず，これまでに可能となった高分子材料による細胞機能制御を**図3**に示します。第1には，材料への生物活性の付与を行うことができます。高分子材料に細胞接着性を付与できることは古くから知られていましたが，成長因子や**サイトカイン**を固定化することにより，成長や分化といった生物学的な高次な

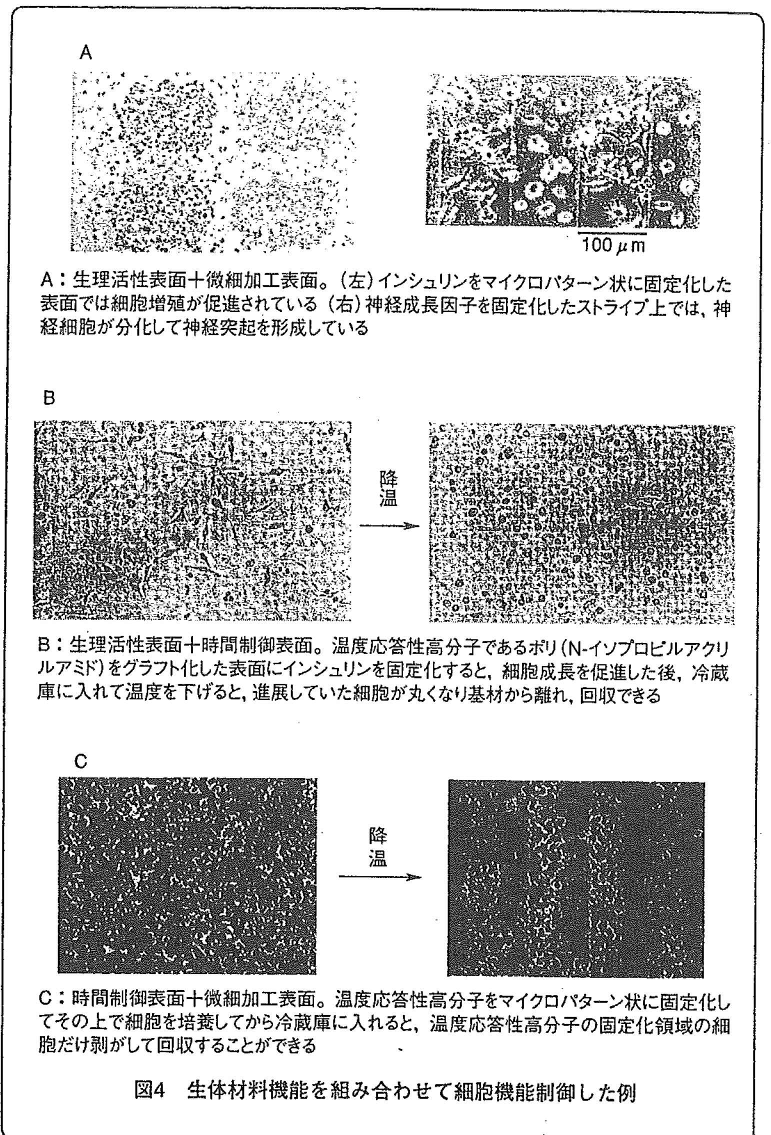
キーワード

サイトカイン｜細胞によって産生され，細胞間の情報伝達を担うタンパク質または糖タンパク質の総称。さまざまなサイトカインに対して，その受容体（レセプター）が存在し，その結合により，細胞の増殖，分化，分泌，アポトーシス，運動など多様な機能が制御される。生体内では，異なったサイトカインが1つの細胞に働きかけ，1つの生理活性を発揮するようなサイトカイン・ネットワークが形成されている。

機能も制御可能であることがわかってきました。第2には，最近盛んになった微細加工技術を用いて，空間的に細胞を特定領域に培養することができるようになりました。第3には，刺激応答性の材料を使って，酵素分解をせずに細胞を回収する技術も開発されてきています。

さらに，これら3つの基礎的な材料機能を複合させ，機能の異なる細胞をマイクロパターン状に培養したり，細胞増殖を促進して大量に培養してから刺激応答で回収したりするなど，新しい原理の材料が開発されてきています（図4）。

そして，このような原理を応用した再生医療への試みが行われています。第1は，幹細胞体外培養基材への応用で，第2はティッシュ・エンジニアリングのスキャホールド（足場）への応用です。



A：生理活性表面＋微細加工表面。（左）インシュリンをマイクロパターン状に固定化した表面では細胞増殖が促進されている（右）神経成長因子を固定化したストライプ上では，神経細胞が分化して神経突起を形成している

B：生理活性表面＋時間制御表面。温度応答性高分子であるポリ（N-イソプロピルアクリルアミド）をグラフト化した表面にインシュリンを固定化すると，細胞成長を促進した後，冷蔵庫に入れて温度を下げると，進展していた細胞が丸くなり基材から離れ，回収できる

C：時間制御表面＋微細加工表面。温度応答性高分子をマイクロパターン状に固定化してその上で細胞を培養してから冷蔵庫に入れると，温度応答性高分子の固定化領域の細胞だけ剥がして回収することができる

図4　生体材料機能を組み合わせて細胞機能制御した例

3. 幹細胞の体外増幅用材料

幹細胞培養は，研究上は確立されている場合もありますが，ヒト幹細胞の培養になると，ほとんど応用的な研究が進んでいません。それは，一般に幹細胞は培養が難しく，単独での培養が困難なためで，**保育細胞**，**フィーダー細胞**と呼ばれる細胞を共存させる必要があるためです。研究用の場合，たとえ培養する幹細胞がヒト由来であっても，保育細胞として異種細胞由来（多くはマウス）細胞を共存させて行われていました。しかし，このような異種細胞との混在のもとで培養されたヒト細胞を医療に用いる場合は，未知の病原体（ウイルスやプリオンなど）の心配があります。幹細胞培養を異種動物由来細胞の共存なしに可能にすることは，再生医療の展開のための重要な課題の一つとなっています（図5）。このためには，保育細胞の機能を模した表面，あるいはまったく異なる発想からなる表面を人工的に作成して，幹細胞の培養が可能になるようにする必要があり，鋭意開発が行われています。

4. ティッシュ・エンジニアリング用材料

造血幹細胞や神経幹細胞は，細胞1個1個のまま細胞医薬のように用いて治療に使うことが

保育細胞，フィーダー細胞｜単独では生存・培養できない細胞を培養する場合に，あらかじめ保育細胞あるいはフィーダー細胞を培養しておく。特に，フィーダー細胞は培地中に不足する栄養分や増殖因子を補給する役割を担う目的で共存させる場合に，保育細胞は，培地中への補給だけでなく，培養したい細胞に直接接触して働く場合の意味合いも含む。

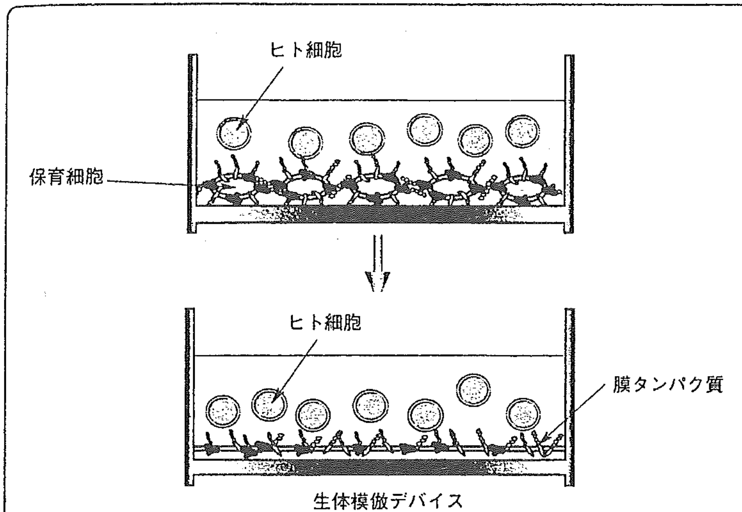


図5　幹細胞の培養

従来のES，造血幹細胞培養では，異種動物由来細胞が保育細胞として使われてきた。病原体などの問題があるため，保育細胞を必要としない人工保育層の構築へ向けた研究が行われている。例えば，保育細胞膜に存在する膜タンパク質を固定化した材料が考えられている。

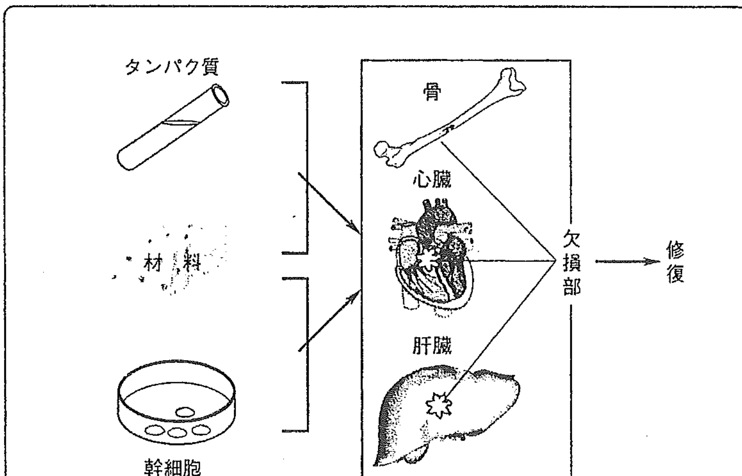


図6　2次元マトリックスを利用した試み

生分解性の3次元マトリックスと細胞成長因子，生分解性の3次元マトリックスと幹細胞とを組み合わせて，生体の欠損部位を補填し，修復を図ることが行われている。

できますが，皮膚や骨のような組織，心臓や肝臓のような臓器の再生には3次元構造を構築する必要があります。そのためには，ティッシュ・エンジニアリングが必要になってきます。現在，3次元マトリックスに成長因子やサイトカインのようなタンパク質を複合化し，生体組織を活性化させて細胞浸潤を促進する材料の開発や，組織細胞や幹細胞を組み込んだ多孔性生分解性材料を生体に埋め込んで欠損部を修復しようとする取り組みが行われています（図6）。3次元構造形成には，さまざまな生分解性高分子，タンパク質，細胞の3つの組み合わせが鍵となります。生体組織は単調に同一の機能の細胞が集合したものではありません。さまざまな機能を担う細胞を有機的に組織するシステムが必要になります。そして生体組織が修復された後は，分解されてなくなってしまうようなマトリックスが望まれています。

最近では，高分子材料を埋め込み用のマトリックスとして使うのではなく，図3の刺激応答性材料上に細胞だけからなる細胞シートを作り，シートのまま剥がして生体修復に使う方法なども開発されてきています。

このように再生医療は高分子材料を使って進歩しています。医学・生物学の領域では幹細胞の機能解明が急速に展開しています。再生医療材料の研究開発にも，これらの知見を生かして，今後さらに高性能化が図られるものと期待されます。

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研究内容：
（財）神奈川科学技術アカデミーの伊藤「再生医療バイオリアクター」プロジェクトにて，再生医療へのバイオマテリアルの応用を研究中。この他に，新しいマイクロアレイ・チップの創成やコンビナトリアル・バイオエンジニアリングについても研究を進める。

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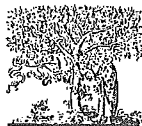
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Biomaterials 27 (2006) 2502–2506

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Technical note

Photoimmobilized array of panel cells for assay of antibodies

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Received 8 October 2005; accepted 21 November 2005

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Received 8 October 2005; accepted 21 November 2005

Abstract

Antibodies in blood are checked with panel blood cells before blood transfusion. In this investigation, for the first time, a panel cell-microarray was prepared by using a photoimmobilization method. Different types of red blood cells were microarrayed on a plate. A water-soluble photoreactive polymer as a matrix was synthesized by the coupling reaction of azidoaniline with poly(2-methacryloyloxyethylphosphorylcholine-*co*-methacrylic acid). The polymer was mixed with cells and the mixtures were microspotted on substrate and photoirradiated after drying in air. For the antibody assay, monoclonal antibodies or human serum was added to the cell-arrayed plate and adsorbed antibodies were detected by horseradish peroxidase-labeled secondary antibody, which recognized the adsorbed antibodies. Antibodies specifically adsorbed on the immobilized cells as expected. The aggregation method has been available for this type of assay, but extensive experience was needed to apply it correctly. The method using a cell array will be useful for antibody detection.
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Keywords: Adsorption; Cross-linking; Phosphorylcholine; Protein adsorption

1. Introduction

Microarray technology has become a crucial tool for large-scale and high-throughput biological science and technology. It allows fast, easy, and parallel detection of thousands of addressable elements in a single experiment under exactly the same conditions [1–3]. DNA microarray approaches have demonstrated a rapid and economic way to interpret gene functions [4,5]. In recent years, there have been considerable achievements in preparing small-molecule arrays [6,7], peptide arrays [8–11], protein arrays [12–15], polysaccharide arrays [16–18], antigen arrays [19–21], antibody arrays [22–27], and tissue arrays [28,29].

Although the preparation of the microarray is important, no universal immobilization method has been developed. Noncovalent immobilization (physical adsorption) or standard chemical coupling reactions (using amino or carboxyl groups in the materials) were usually used. However, the former is not suitable for stable immobilization and the latter is limited by the structure of the immobilized materials. Therefore, Ito et al. developed a photoimmobilization method for the preparation of a microarray chip [30]. Using this method, any material can be immobilized on a substrate by the use of radical reactions. Ito et al. microarrayed proteins using photoreactive polymers as the matrix on a tissue culture polystyrene plate [30,31]. In the present study, cells were microarrayed using this method. Although a microarray of cultured cell lines for antibody detection of cell surface proteins has been reported by Schwenk et al. [32], the use of a cell microarray for clinical analysis has not been reported.

Here, the assay of antibodies in blood was chosen as the target of the cell array. Usually, A-type blood does not have anti-A antibody. However, irregular antibodies are sometimes produced by previous transfusion or pregnancy. Blood that contains irregular antibodies cannot be used for

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doi:10.1016/j.biomaterials.2005.11.030

transfusion, because they aggregate with the patients' cells. Therefore, the presence of irregular antibodies is usually checked before transfusion. However, as the check is performed by an aggregation assay of blood cells, it is a time-consuming process that requires some skill to judge a positive result. If the cell array method is realized, washing and staining processes can be automated and the assay system will be conveniently performed. Recently, an automatic analysis using an allergen-microarray was reported [21].

2. Materials and methods

2.1. Preparation of photoreactive polymer

Photoreactive polymer was prepared as previously reported [33]. A copolymer consisting of 2-methacryloyloxyethyl phosphorylcholine (90 mol%) and methacrylic acid (10 mol%) was obtained from NOF Co. (Tokyo, Japan); it is referred to as PMAc. The molecular weight of PMAc, as measured by gel permeation chromatography, was 2.2×10^5. Modification of PMAc was performed as follows. 4-Azidoaniline (12.44 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (17.47 mg), purchased from Dojindo Lab. (Kumamoto, Japan), were dissolved in 2 mL of PMAc solution (5 wt%); 98 mL of water was added to the solution. The solution was left to stand for 24 h. After reaction, the product was dialyzed with a dialysis cassette (PIERCE, Rockford, IL, USA) until ultraviolet absorption confirmed no further release of azidoaniline through the cassette. The resultant solution was freeze-dried. The azidophenyl-derivatized PMAc is referred to as Az-PMAc. Elemental analysis indicated that the amount of azidophenyl group in Az-PMAc was 6%.

2.2. Preparation of cell array

Cell array was carried out as follows. Cells carrying M, N, A, B, AB, O, or Rh (D) antigen were chosen and nine kinds of panel cells, M +, N +, A; D +, B; D +, AB; D +, O; D +, O; D–, D +, and D– were employed. The symbols + or – mean the presence or absence of antigen on the cells, respectively. The cells were washed twice with phosphate buffered saline (PBS). After the washed cells were incubated in 2 vol% glutaraldehyde solution for 15 min or left untreated, they were mixed with 1 wt% of photoreactive polymer. The suspension (0.5 μL) was spotted on a tissue culture polystyrene dish (diameter 35 mm, 3000-035X) purchased from Iwaki Glass Co., Ltd. (Chiba, Japan). After drying in air, the spot was immobilized by UV irradiation. The immobilized cell spot was washed three times with PBS containing 0.1 wt% Tween 20.

2.3. Binding assay of antibodies

Mouse antibodies or human sera were prepared as follows. In the case of anti-M, -A, -B, or -GPA, mouse (BALB/c) was immunized with each human erythrocyte and the immunized spleen cell was fused with a myeloma cell (NS-1). The fused cell was repeatedly cloned, each antibody-producing cell was established, and each antibody was collected from the cell. In the case of anti-D antibody, B lymphocyte isolated from humans carrying anti-D antibody was transformed with Epstein-Barr virus, and the transformed cell was cultured and fused with the myeloma cell. The fused cell was repeatedly cloned, an antibody-producing cell was established, and the antibody was collected. In the case of human sera, they were collected from healthy volunteers.

Binding assay was performed as follows. PBS containing anti-M, -A, -B, -D, or -glycophorin A (GPA) mouse monoclonal antibody (53, 62, 57, 58, and 86 mg/L, respectively), or human serum of A, B, O, or AB was added to the cell array plate and incubated at 22 °C for 2 h. After incubation, the plate was washed with PBS containing 0.1 wt% Tween 20. Subsequently, the plate was incubated with a PBS containing anti- or -human whole antibodies IgG, linked with horseradish peroxidase (Amersham Bioscience Co., Piscataway, NJ, USA) at 22 °C for 1 h. After washing three times with PBS containing 0.1 wt% Tween 20, ECL reagents (Amersham Bioscience Co.) were added. The plate was analyzed using a chemiluminescence image analyzer, $n = 4$.

3. Results and discussion

First, the M + cells were fixed with glutaraldehyde and mixed with PMAc or Az-PMAc, and the mixture was arrayed on a tissue culture polystyrene plate for the binding assay of monoclonal anti-M antibody. It was found that anti-M antibody bound to the spot of M + cells immobilized with Az-PMAc, but not with PMAc. Because the cells were immobilized by the photocrosslinking reaction, the cells were not considered to be washed out during the binding assay. This result indicates that photoimmobilization was important for the binding assay.

Second, the binding of anti-M and -GPA antibody to the glutaraldehyde-fixed and photoimmobilized M + or N + cells was investigated. Anti-GPA was used as a control and it recognized both immobilized M + and N + cells. On the other hand, anti-M antibody bound to immobilized M + cells, but not to N +. These results demonstrate that the recognition of antibody with the cells was specific. Dilution of the cells for the immobilization reduced the amount of bound antibodies.

Third, the effect of glutaraldehyde treatment on antigens locating on panel cells was investigated. Fig. 1 shows that anti-A and -B antibodies bound to A and B cells, respectively. Anti-D antibody bound to D + -type cells, but not to D–-type cells. In this case, because all the A, B, and AB cells have D antigen, anti-D antibody bound to all the D + panel cells. However, a comparison of Figs. 1a and b shows that the amount of anti-D antibody bound to cells treated with glutaraldehyde (Fig. 1a) was lower than for those that were not treated (Fig. 1b). D antigen is a membrane protein with a complex structure and anti-D antibody recognizes the three-dimensional structure of D antigen. For recognition of D antigen, glutaraldehyde treatment was not appropriate. Considering that glutaraldehyde fixation affects the molecular recognition, the fixation was not carried out before photoimmobilization for further experiments.

Fig. 2 shows photographs indicating monoclonal antibodies bound to spotted panel cells that were photoimmobilized with Az-PMAc. Anti-A and -B antibodies bound to the immobilized A; D + and B; D + cells, respectively. In addition, the antibodies bound to the AB cells. On the other hand, in this case, because O; D– cells had no D antigen, no reaction of anti-D antibody was found. These phenomena correspond to the result of an aggregation test for conventional blood types.

Finally, the array assay was used for human serum, as shown in Fig. 3. O-type serum reacted with A, B, and AB type cells. A- and B-type serum reacted with B and A cells,

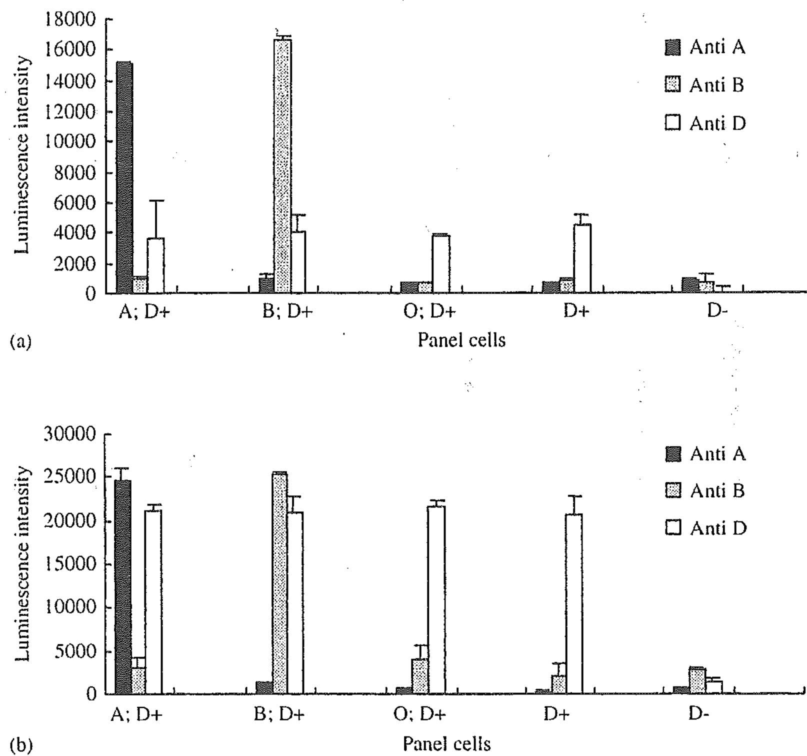

Fig. 1. Effect of glutaraldehyde fixation of panel cells on binding of antibody. (a) Glutaraldehyde-fixed panel cells were arrayed and (b) nonfixed panel cells were arrayed. Binding of anti-A (black), anti-B (gray), and anti-D (white) antibodies onto the cells.

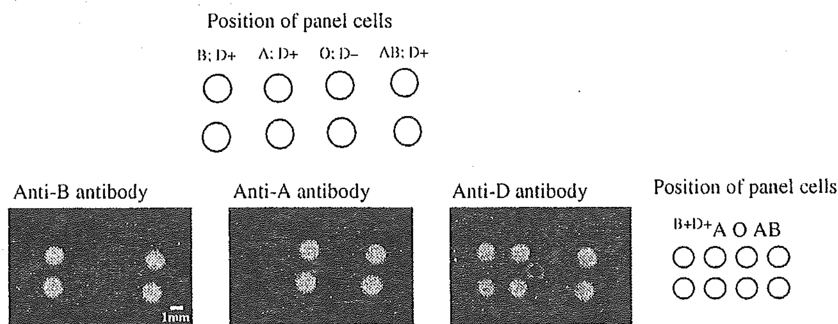

Fig. 2. Detection of chemiluminescence of antibodies bound on photoimmobilized panel cells array.

respectively, about 2-fold as high as with other cells. This is because A- and B-type serum have anti-B and -A antibody, respectively. AB-type serum did not react with either type of cell, because it has no antibody. Because O-type serum has both anti-A and -B antibodies, the panel cells except for O; D+ cells were stained. These phenomena correspond to the result of an aggregation test for conventional blood types.

Recently, Zhang et al. reported the capture of red blood cells by blood group antibodies (anti-A and -B) [34]. Conversely, this study has demonstrated that cells can be arrayed and immobilized on a plate and that the immobilized array was successfully employed for antibody detection, although some treatment of cells, for example glutaraldehyde fixation, depends on the characteristics of the antigen. The photoimmobilization method will be useful in the array technique as an "everything array method", even for cells.

4. Conclusions

A panel cell-microarray was prepared by using a photoimmobilization method. A photoreactive polymer was mixed with different types of red blood cells and the mixture was microspotted on substrate and photoirradiated. By this method the cells were immobilized on the susbstrate. Human serum was added to the cell-arrayed plate and adsorbed antibodies in the serum were detected

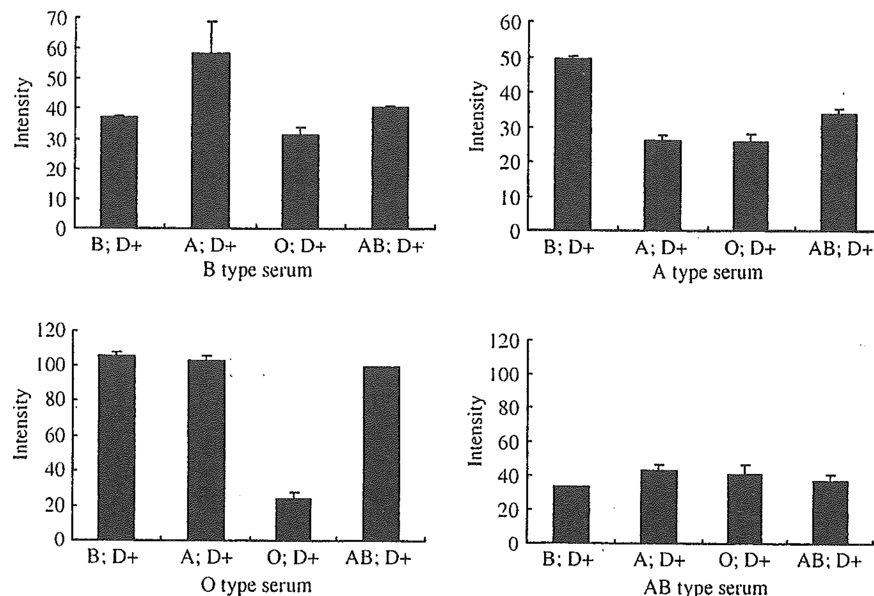

Fig. 3. Detection of chemiluminescence of antibodies in the serum of some blood types bound on photoimmobilized panel cells array.

as expected. The method using a cell array is useful for antibody detection because of its convenient multiple analysis of antibodies in human serum.

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Allergology International. 2005;54:627-631

ORIGINAL ARTICLE

A Photo-immobilized Allergen Microarray for Screening of Allergen-specific IgE

Kunio Ohyama[1], Kaoru Omura[1] and Yoshihiro Ito[1,2]

ABSTRACT

Background: We developed an *in vitro* system to diagnose allergy using an allergen microarray and photo-immobilization technique. Photo-immobilization is useful for preparing the allergen microarray because it does not require specific functional groups of the allergen and because any organic material can be immobilized by a radical reaction induced by photo-irradiation.
Methods: To prepare the plates, allergen solutions were mixed with polymer and a bis-azidophenyl derivative, a photo-reactive cross-linker, the mixtures were micro-spotted on the plate, and the droplets were dried. The plate was irradiated with an ultraviolet lamp for immobilization. For the assay, human serum was added to the microarray plate.
Results: Allergen-specific immunoglobulin E (IgE) adsorbed on the micro-spotted allergen was detected by peroxidase-conjugated anti-IgE antibody. The chemiluminescence intensities of the substrate decomposed by the peroxidase were detected with a sensitive CCD camera.
Conclusions: All allergens were immobilized by this method and used to screen allergen-specific IgE.

KEY WORDS

allergen microarray, allergen-specific IgE, allergy diagnosis, chemiluminescence, photo-immobilization

INTRODUCTION

To study allergic reactions, it is important to develop test systems to measure immunoglobulin E (IgE) concentration in serum samples. The first radioallergosorbent test (RAST) to detect allergen-specific IgE in serum was described in 1967.[1] Subsequent tests replaced the radioactive labels used in the RAST with various procedures such as the chromogenic-enzyme immunoassay (EIA) or fluorescence-enzyme immunoassay (FEIA). However, few of these have become routine methods in the diagnosis of allergy in research and clinical practice.[2-7] The most common *in vitro* technique used in the clinical settings is the Pharmacia CAP System (PCS) to measure total and allergen-specific IgE (specific IgE FEIA, Pharmacia, Uppsala, Sweden). Other methods such as the FAST FEIA (MAST Diagnostica, Reinfeld, Germany) and HYTEC EIA (Hycor Biomedicals, Kassel, Germany) are available commercially.[8] Some methods are based on liquid-phase inhibitor assays (e.g., AlaSTAT, DPC Biermann, Los Angeles, CA, USA) or multiallergen-coated nitrocellulose strips (e.g., IgEquick, Teomed AG, Greifensee, Switzerland; CMG Immunodot, Trimedal AG, Brüttisellen, Switzerland).[9,10] The CAP System contains a cellulose polymer densely conjugated with allergen extracts or recombinant allergens.

Although it is possible to measure a multitude of allergen-specific IgEs by immunoassays in the patient's blood, these tests are expensive, time consuming, and some need a high volume of reagents and serum. An increasing number of patients are experiencing immediate-type allergic diseases, such as allergic rhinoconjunctivitis, atopic eczema, and food and drug allergies.[11] It is desirable to develop a fast and economic screening technology to detect allergen-specific IgE in serum samples that allows the simultaneous analysis of hundreds of allergens in a single run. Multiallergen dipstick tests were a first step in

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Received 8 February 2005. Accepted for publication 20 June 2005.
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Table 1 UniCap data of sera (IU/ml)

Allergen	M-14	SIC311276	AHP9580	AHP9549	SIC31181
Japanese cedar	8.79	< 0.34	< 0.34	9.37	30.9
Dermatophagoides pteronyssinus	0.88	36.4	0.63	4.50	12.8
Orchard grass	< 0.34	< 0.34	65.1	99.9	≥ 100
Cow milk	≤ 0.34	< 0.34	< 0.34	14.7	37.9
Egg white	≤ 0.34	0.52	< 0.34	11.4	33.1

The values corresponding to the chemiluminescent spots of Figure 4 are underlined.

the miniaturization and cost savings of such techniques,[10,12] but most could not be run automatically. Microarrays produced with spotting devices are another strategy to miniaturize such tests, which allow proteins to be immobilized in the lower nanoliter range on defined positions on a surface. The first experimental microarray system for allergy diagnosis was reported in 2000,[13] and an allergen microarray based on fluorescence detection was published in 2002.[14] Fall *et al.* reported recently on an application of the parallel affinity sensor array (PASA) technology that automatically performs allergy diagnosis.[2] Purified recombinant and natural allergens and allergen extracts were immobilized on glass slides to detect allergen-specific IgE. However, not all allergens were immobilized by the technique because specific functional groups are needed by the allergens.

We have developed a photo-immobilization method to apply the microarray to various materials including proteins and cells.[15,16] We used this photo-immobilization technique to prepare a microarray of allergens. The advantages of the photo-immobilization method are that it is not limited by functional groups and that it can immobilize any organic material in any organic substrate.

METHODS

REAGENTS AND CHEMICALS

Plates for microarray (polystyrene slides, 2.5 cm × 7.6 cm × 0.5 mm) were cleaned using ethanol with sonication for 15 minutes at room temperature. The washed polystyrene slides were dried and stored. The raw allergen materials, Japanese cedar, orchard grass, *Dermatophagoides pteronyssinus*, cow milk and egg white, were purchased from Allergon (Ängelholm, Sweden). The polyclonal affinity-purified horseradish peroxidase (HRP) -labelled goat anti-human IgE antibody was purchased from Serotec Ltd (Oxford, UK). The ECL Advance Kit for HRP was purchased from Amersham Biosciences UK Ltd (Buckinghamshire, UK). 4,4'-diazido-stylbene-2,2'disulfonic acid, disodium salt (BIS), polyethylene glycol methacrylate (molecular weight, 526 Da), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co (Milwaukee, WI, USA). Sera containing allergen-specific IgE for Japanese cedar (M-14), *Dermatophagoides pteronyssinus* (SIC311276), orchard grass (AHP9580), cow milk (AHP9549), and egg white (SIC31181) were purchased from Uniglove Research Corp. (Rivera, CA, USA). The reference measurements were performed with the UniCap System (CAP specific IgE-FEIA, Pharmacia, Uppsala, Sweden) and the data are shown in Table 1.

SYNTHESIS OF PEG-350

The polymer matrix carrying polyethylene glycol in the side chains (PEG-350) was prepared as follows. Polyethylene glycol methacrylate (molecular weight 350 Da, 7.0 *g*) was dissolved in ethyl acetate (80 mL) and bubbled with nitrogen gas for 30 seconds. Azobisisobutyronitrile (46.0 mg) was added to the solution, which was then allowed to stand for 6 hours at 60℃. The solution was concentrated and added to diethyl ether. A viscous solid was obtained after stirring. The precipitation procedure was repeated four times and the final precipitate was dried *in vacuo*. The yield was 1.57 *g* (22.4%).

PREPARATION OF ALLERGENS

To prepare the allergen extracts, 5% raw allergen material (w/v) was suspended in 0.05 M phosphate buffer (pH7.4) for 2 hours at 4℃. The supernatant was collected and filtered through a 0.45 µm cellulose acetate membrane (Sartorius, Göttingen, Germany). The supernatant was dialyzed against water for 24 hours and then lyophilized.

PHOTO-IMMOBILIZATION OF ALLERGEN

The principle of immobilization is illustrated in Figure 1. We propose that BIS works as a photo-reactive cross-linker to immobilize the allergen with PEG-350 and that photo-irradiation causes the cross-linking reaction to occur between allergen and allergen, allergen and PEG-350, allergen and the plate surface, and PEG-350 and the plate surface.

The extracted allergens were dissolved in deionized water at various concentrations (0.625−40 mg/mL). The allergen solutions were mixed with an aqueous solution of BIS (0−0.5 mg/mL), and PEG-350 (0.25 mg/mL) at a 2:1:1 volume ratio. The mixtures were micro-spotted (50 nL) with the microarray spotter (PixSis-4500, Cartesian, Irvine, CA, USA) on

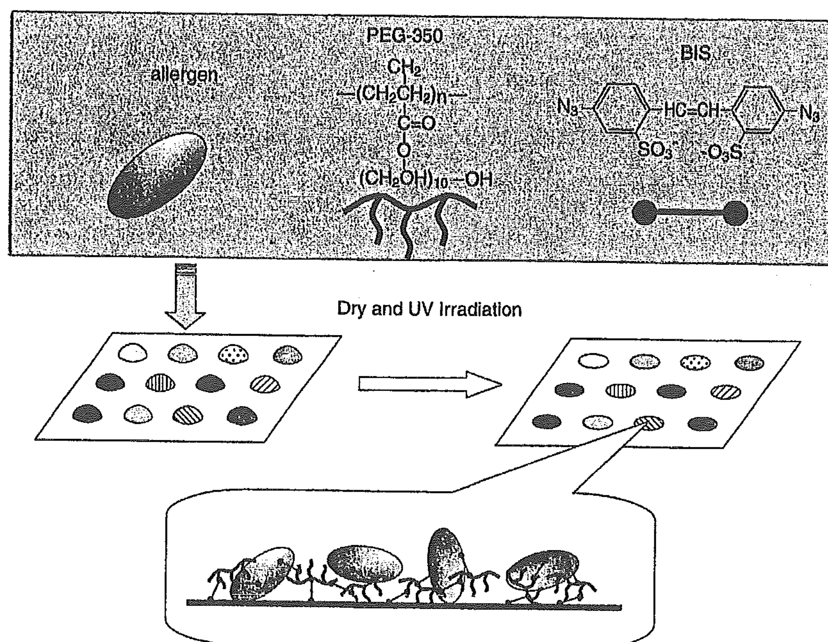

Fig. 1 Illustration of the photo-immobilization method. Abbreviations: PEG-350, polymer carrying polyethylene glycol in the side chains; BIS, 4,4'-diazido-stilbene-2,2'-disulfonic acid disodium salt.

the plate and the droplets were dried. The microarrayed plate was irradiated with an ultraviolet lamp (300–400 nm, Nippo Electric Co. Ltd. FL15BLB) for 7 minutes. Finally, the allergen-immobilized plates were rinsed with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (the washing buffer), and stored until use in a refrigerator.

MICROARRAY ASSAY PROCEDURE

The allergen-immobilized plates were incubated with serum (30 μL) for 1 hour at room temperature with shaking in a chamber. The plate was washed with 30 mL of the washing buffer for 3 minutes in a chamber. HRP-conjugated anti-human-IgE antibody (diluted 1:100 with PBS-10% BSA) was loaded on the microarray plates and the plates were incubated for 1 hour at room temperature with shaking in a chamber. Finally, the substrate solutions (ECL Advance Kit) were added to the plates and the plates were incubated for 3 minutes at room temperature. The chemiluminescence intensities of each micro-spot were measured for 30 seconds with a cooled CCD camera system (AE-6960 Light Capture, ATTO Corp., Tokyo, Japan).

RESULTS

PHOTO-IMMOBILIZATION

To examine the photo-immobilization method, we immobilized orchard grass under various conditions. No chemiluminescence was observed when allergen was not contained in the micro-spot (data not shown). This result indicates that neither BIS nor PEG-350 induces non-specific adsorption of antibodies. Although neither BIS nor PEG-350 immobilized allergen, some intensity of chemiluminescence was observed (Fig. 2). Non-specific (physical) adsorption of allergen was considered to occur on the plate surface. In contrast, micro-spotting with both BIS and PEG-350 produced maximum intensity (Fig. 2). This result indicates that the allergen was stably immobilized on the plate surface; BIS caused cross-linking between allergen and allergen, and between allergen and the plate surface, and thus immobilized allergen on the plate and PEG-350 was considered to increase the amount of immobilized allergen by entrapping in the crosslinked matrix

CALIBRATION CURVE

Using the condition producing the highest chemical luminescence intensity, we produced calibration curves for orchard grass, Japanese cedar, *Dermatophagoides pteronyssinus*, cow milk, and egg white, which are shown in Figure 3. The coefficients of variation (CV) were <10.0%, although the pattern of the calibration curve differed between the allergens.

ASSAY USING MULTIPLE MICRO-SPOTTED PLATES

Figure 4 shows the chemiluminescence image produced by IgE adsorbed on micro-spotted orchard grass, Japanese cedar, *Dermatophagoides pteronyssinus*, cow milk, and egg white. Although Fall *et al.*[2] could not immobilize all the antigens they studied,

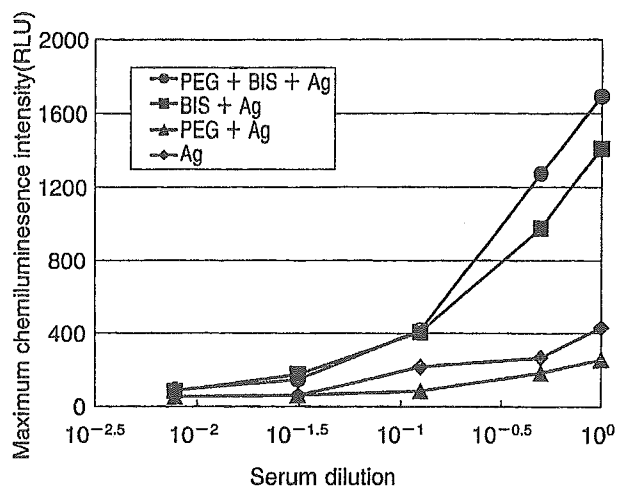

Fig. 2 Adsorption of orchard grass-specific IgE on the surface micro-spotted with ◆, allergen only; ▲, allergen with PEG-350; ■, allergen with BIS; and ●, allergen with PEG-350 and BIS. The concentrations of micro-spotted allergens were 2.5 mg/mL for orchard grass allergen, 2.5 mg/mL for PEG-350, and 0.125 mg/mL for BIS. Serum was diluted 1-, 2-, 8-, 32- and 128-fold with PBS-4% BSA. $N = 4$.

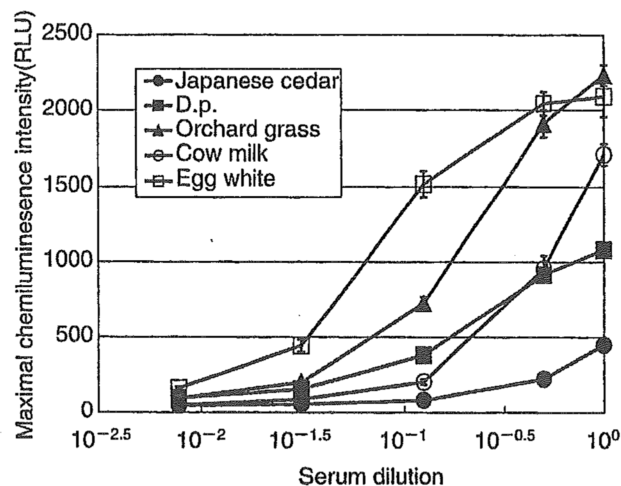

Fig. 3 Calibration curve of allergen-specific IgE on micro-spotted allergens. Orchard grass (2.5 mg/mL), Japanese cedar (10.0 mg/ml), *Dermatophagoides pteronyssinus* (20.0 mg/ml), cow milk (10.0 mg/ml), and egg white (10.0 mg/ml) were micro-spotted and photo-immobilized with BIS (0.125 mg/mL) and PEG-350 (2.5 mg/mL). Serum was diluted 1-, 2-, 8-, 32- and 128-fold with PBS-4% BSA. Data are presented as means ± SD ($N = 4$).

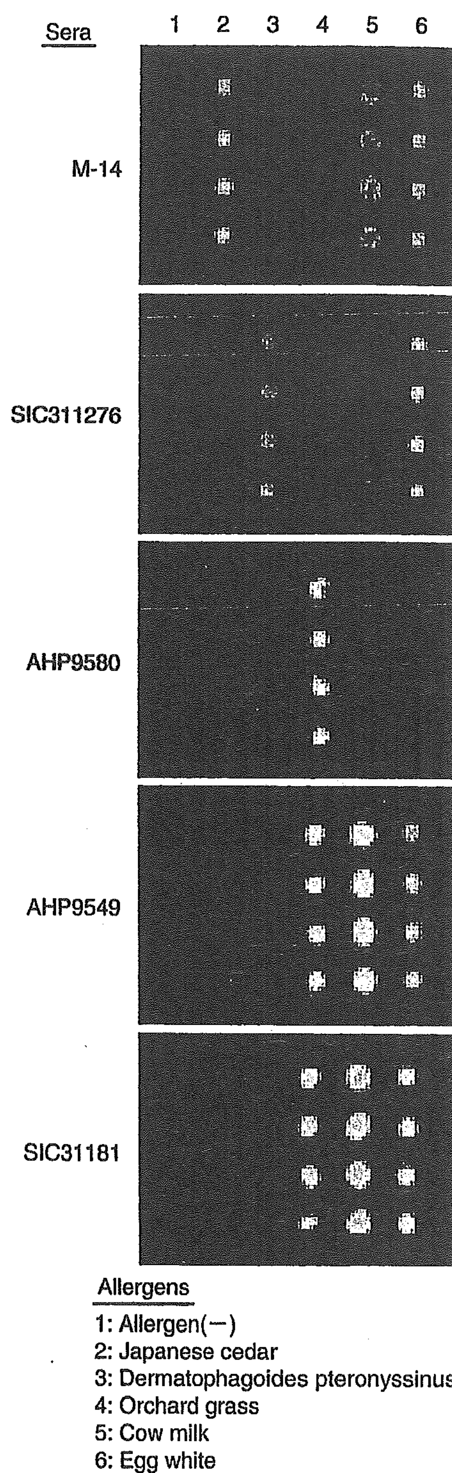

Fig. 4 Chemiluminescence image of IgE adsorbed on micro-spotted Japanese cedar, *Dermatophagoides pteronyssinus*, orchard grass, cow milk, and egg white.

our results show that all antigens were immobilized and that we observed chemiluminescence of each allergen spot by adsorption of IgE from the corresponding serum. In addition to the IgE, which should be contained in serum according to the supplier, Figure 4 shows the allergic reactions between the various allergens and serum. M-14 and SIC311276 each produced an allergic response to cow milk and egg white. AHP9549 produced an allergic response to or-

chard grass and egg white, and SIC31181 produced an allergic response to orchard grass and cow milk.

DISCUSSION

The photo-immobilization method uses the radical reaction for cross-linking, making it possible to immobilize any organic materials independently of functional groups of chemicals or proteins.[15-17] It is easy and convenient to prepare multiple micro-spots by the same method. We have previously immobilized various types of proteins to analyze cell adhesion and panel cells to detect antibody in blood.[15,16,18]

One additional characteristic of the assay is that the orientation of immobilized molecules is random. This property is suitable for the allergen microarray because various sites of immobilized molecules are exposed from the surface, enhancing recognition by polyclonal IgE (Fig. 1). The ability to measure the amount of IgE in the diluted serum, shown in Figure 3, indicates that this property enhances the assay's sensitivity.

Since standardization was not completely performed, the chemiluminescent spots were not completely in accord with the data in Table 1. However, in each sample higher values of UniCap measurement corresponded to the chemiluminescent spots. This indicates the adequacy of the present measurement.

Another characteristic is the small amount of serum required for the microarray assay. Usually 300 μL of serum is required to analyze five different allergens in the conventional one-to-one assay. However, this system required one-tenth that amount (30 μL) to analyze five allergens. Integrating the microarray density will increase the number of allergens that can be analyzed.

Our study demonstrates that this photo-immobilization technique is useful for screening of allergen-specific IgE to diagnose allergy. The ability to adapt this technique to immobilize any organic material has potential applications in developing assays to analyze auto-immunity and infection.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Japan Science and Technology Agency.

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Culture of human umbilical vein endothelial cells on immobilized vascular endothelial growth factor

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Culture of human umbilical vein endothelial cells on immobilized vascular endothelial growth factor

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Received 12 May 2004; revised 6 January 2005; accepted 3 February 2005
Published online 20 July 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.30360

Abstract: Vascular endothelial growth factor (VEGF) was immobilized on substrata in photoreactive gelatin to control the adhesion and growth of vascular endothelial cells. The gelatin and VEGF were mixed in water and cast on a polystyrene dish or a silane-coated glass plate. The surface was then photoirradiated in the presence or absence of a photomask and washed. Toughness of the immobilized material was confirmed by ethanol treatment. Human umbilical vein endothelial cells (HUVECs) grew on the immobilized VEGF but not on a nontreated surface. Growth of HUVEC increased significantly with an increase in the amount of immobilized VEGF, and the effects were inhibited by treatment with anti-VEGF antibody. Thus, immobilized VEGF specifically interacted with HUVECs to permit growth in culture. Micropatterning of HUVEC cultures was also achieved using micropattern-immobilized VEGF. This patterning technique may be useful for the formation of blood vessel networks *in vitro*. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 74A: 659–665, 2005

Key words: immobilization; vascular endothelial growth factor; human umbilical vein endothelial cells; micropatterning

INTRODUCTION

The construction of blood vessels is a fundamental challenge for regenerative medicine. To construct or regenerate organs, formation of a capillary network is essential to transport nutrients and gas into organs, and control of vascular endothelial cell growth is necessary to achieve this *in vitro*. Recently, Koike and colleagues[1] showed that a network of long-lasting blood vessels can be formed in mice by implantation of vascular endothelial cells and mesenchymal precursor cells cocultured in a gel matrix, thus bypassing the need for risky genetic manipulation. Vascular endothelial growth factor (VEGF) is a mitogen primarily acting on vascular endothelial cells. It was purified by Gospodarowicz and colleagues[2] and Ferrara and Henzel[3] from a conditioned medium of bovine pituitary follicular stellate cells, utilizing an endothelial cell proliferation assay to monitor the biological activity. Purified VEGF is a protein of approximately 46 kDa, which dissociates upon reduction into two apparently identical 23 kDa subunits.

Ito and colleagues[4,5] found that biosignal molecules (growth factors and cytokines) immobilized on a solid matrix enhanced cell growth. Since this discovery, various groups have confirmed the biological activity of biosignal molecules immobilized on various matrices.[6–18] Immobilized growth factors transduce a signal for a longer time than do soluble growth factors, and this enhances growth to a greater extent. On the other hand, Ishikawa and colleagues[19] and Hayashi and coworkers[20] synthesized chimeric proteins containing adhesion and growth factors and showed that these enhanced adhesion and growth of cells. Recently, Zisch and colleagues[21] reported that fibrin-bound ephrin-B2 acts as multivalent ligand for endothelial cells. Here, VEGF was photoimmobilized in gelatin, and human umbilical vein endothelial cells (HUVECs) were successfully cultured on the resulting surface. Micropattern-immobilization was employed not only to investigate the effect of immobilized growth factor, whether the cell behavior was affected on the growth factor-immobilized surfaces or not, but also to induce network formation.

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Contract grant sponsor: Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; contract grant number: 14380406
Contract grant sponsor: Uehara Memorial Foundation

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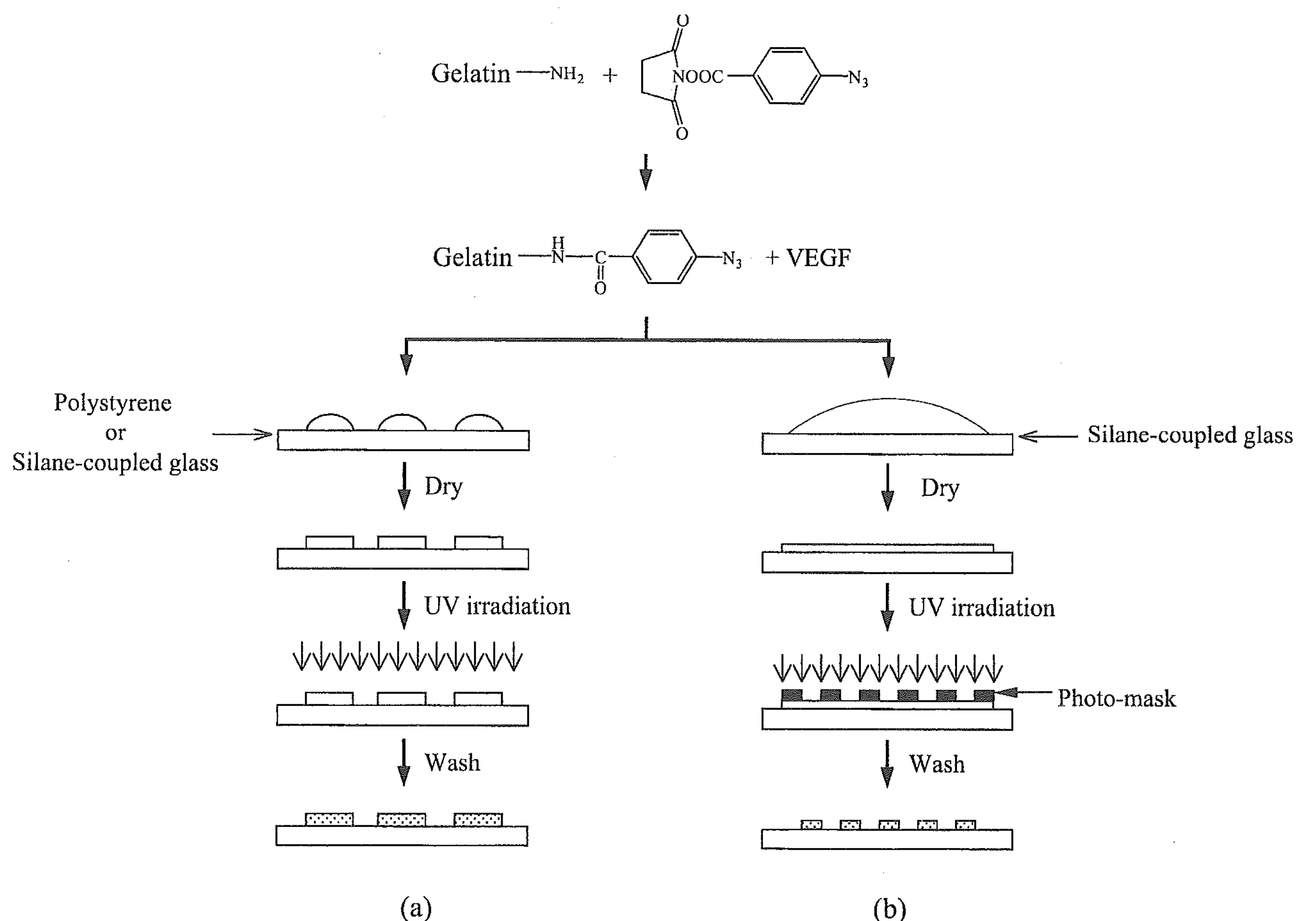

Figure 1. Immobilization of VEGF with photoreactive gelatin. (a) Microspot formation; (b) micropattern formation.

MATERIALS AND METHODS

Materials

Human recombinant VEGF 121, recombinant human VEGF receptor 2 (KDR/Fc) chimera (357-KD/CF), and antihuman VEGF antibody (MBA293) were purchased from R & D Systems, Inc. (Minneapolis, MN). Antihuman Fc antibody (rabbit), antirabbit IgG antibody conjugated with biotin, and streptavidin-bound horseradish peroxidase (HRP) were purchased from Dako Cytomation A/S (Copenhagen, Denmark). DAB was purchased from Dojin Chem, Inc. (Kumamoto, Japan). Gelatin (porcine) was purchased from BD Diagnostic Systems (Sparks, MD). Dicyclohexylcarbodiimide (DCC), dioxane, *N*-hydroxysuccimide, and dimethylformamide (DMF) were purchased from Wako Pure Chem Ind. (Osaka, Japan) and used without further purification. 4-Azidobenzoic acid was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and used without further purification. Polystyrene dishes were purchased from Iwaki & Co., Ltd. (Tokyo, Japan). Octadodecylethoxysilane for surface treatment of glass plate was purchased from Shinetsu Chem. Ind. Co., Ltd. (Tokyo, Japan). HUVECs were purchased from Cambrex (Wakersville, MD).

Synthesis of photoreactive gelatin

Immobilization was performed as reported.[22] Photoreactive gelatin was synthesized as follows. First *N*-(4-azidobenzoyloxy) succinimide was prepared. A solution of DCC (380 mg) was added dropwise to a solution of *N*-hydroxysuccinimide (210 mg) and 4-azidobenzoic acid (300 mg) in dioxane (20 mL), and then cooled in an ice bath while stirring for 12 h. The white solid that formed was filtered off and the solvent was removed under reduced pressure. The yellow residue obtained was crystallized twice from dioxane/diethyl ether.

Gelatin, dissolved in 10 mL phosphate-buffered solution (pH 7.0), was added to a solution of *N*-(4-azidobenzoyloxy) succinimide (25.8 mg) in DMF (20 mL) while being stirred on ice. After a stirred incubation at 4°C for 24 h, the solution was filtered using Millipore ultrafiltration membranes (10,000 molecular weight cutoff). The dialyzed sample was freeze dried and the content of azidophenyl groups in the modified gelatin was calculated from the light absorbance at 270 nm.

Photoimmobilization of VEGF

Immobilization is illustrated in Figure 1. VEGF was mixed with photoreactive gelatin in water (180 μL) and aliquots of

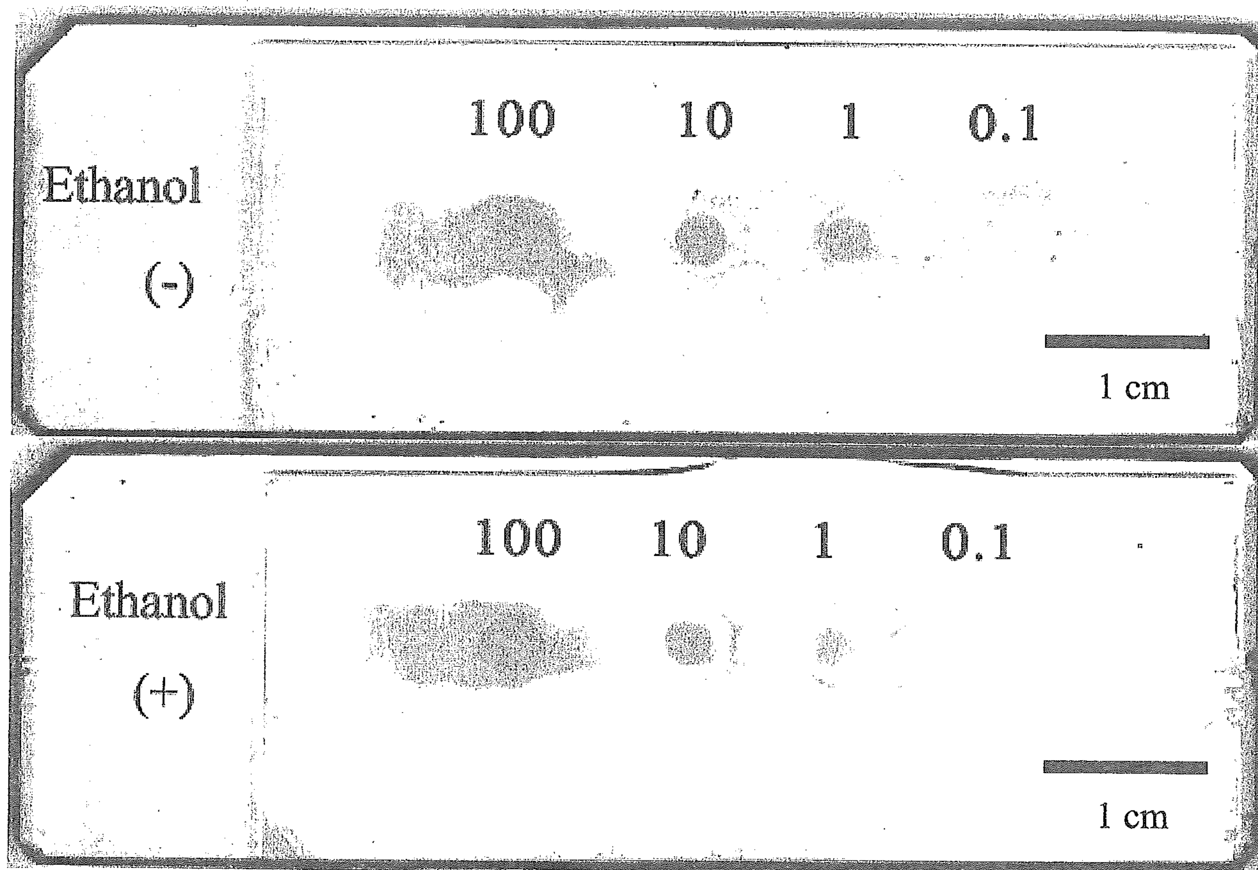

Figure 2. Staining of immobilized VEGF. The immobilized slide glass plate was incubated with recombinant VEGF receptor 2 (KDR/Fc), with anti Fc antibody, with the secondary antibody coupled with horseradish peroxidase, and finally with DAB solution. The plate is shown before (−) and after (+) 70% ethanol treatment. The numbers show the concentrations of VEGF in micrograms per milliliter. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the aqueous solution were dropped onto tissue culture polystyrene dishes or silane-coated glass plates. The silane-coated glass plates were prepared as follows. Plates were incubated in Piranha solution (sulfuric acid: 30% aqueous peroxide solution, 7:3) under sonication for 10 min and left in the solution for 1 h. The glass plate was rinsed with MilliQ water five times and dried *in vacuo* at 50°C for 2 h. The pretreated glass plate was incubated in a solution of octadecylethoxysilane (10 m*M*) in toluene at room temperature overnight. The plate was then washed with toluene and dried *in vacuo* for 1 h.

Each dish or plate coated with photoreactive gelatin plus VEGF was covered with or without a photomask (Nippon Filcon Co. Ltd., Tokyo, Japan) and irradiated using a UV spot Light Source L5662 (Hamamatsu Photonics, Hamamatsu, Japan) from a distance of 5 cm for 10 s (16 mW/cm^2). The dish or plate then was washed repeatedly with distilled water.

Staining of immobilized VEGF

The glass plate with immobilized VEGF was treated with or without 70% ethanol for 10 min at room temperature. Subsequently the plate was washed with phosphate-buffered saline (PBS) three times and incubated in a PBS solution containing 1 μg/mL of KDR/Fc and 0.1% bovine serum albumin (BSA) for 2 h at 37°C. The plate was washed with PBS three times and incubated in a PBS solution containing antihuman Fc antibody (diluted 1:1000) and 0.1% BSA for 1 h at room temperature. The plate was washed with PBS three more times and incubated in a PBS solution containing biotinylated antirabbit IgG antibody (diluted 1:500) and 0.1% BSA for 30 min at room temperature. The plate was washed with PBS three more times and then incubated in PBS containing streptavidin-HRP (diluted 1:700) and 0.1% BSA for 30 min at room temperature. Finally the plate was washed with PBS five times and incubated in DAB solution for 3 min at room temperature.

Cell culture

HUVECs were cultured in EBM-2-MV bullet kits (Cambrex); basal endothelial medium (EBM-2) supplemented with 5% fetal calf serum (FCS), VEGF, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), R3-insulinlike growth factor (R3-IGF-1), hydrocortisone, ascorbic acid, and antibiotics (SingleQuots kit, Cambrex). For

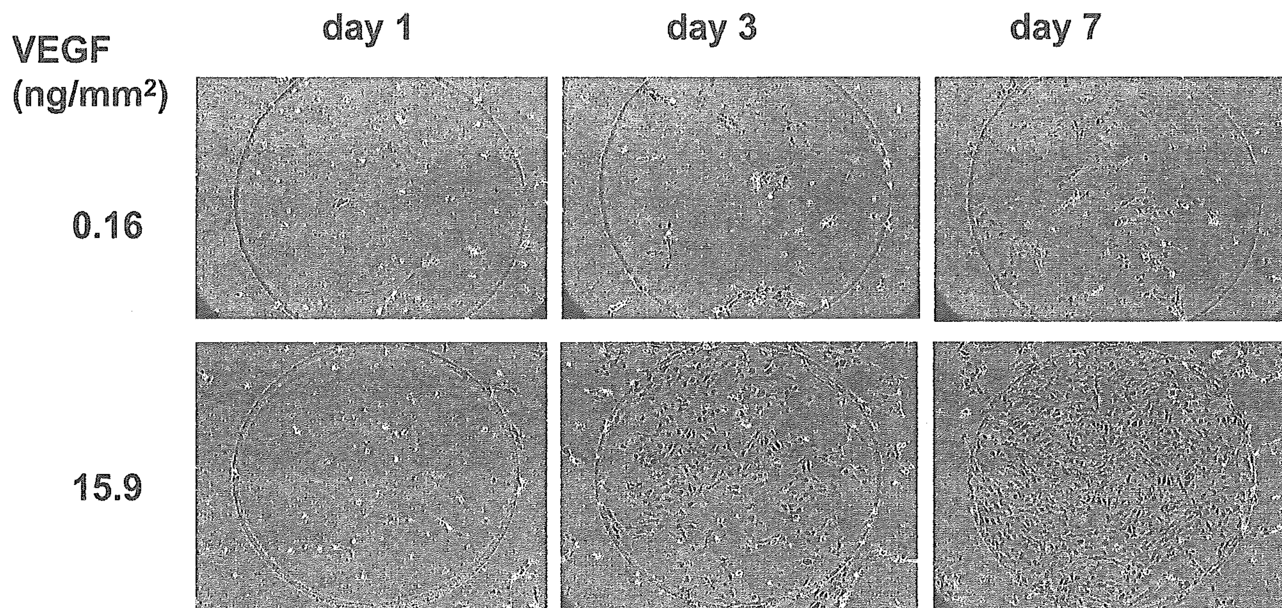

Figure 3. Phase-contrast micrographs of HUVECs cultured on tissue culture polystyrene dishes with different concentrations of immobilized VEGF. The immobilized region is in the center of each photo. The surface concentration of gelatin was 15.9 ng/mm^2.

experiments, they were harvested after trypsinization and washed with EBM-2 medium supplemented with 0.5% FCS and SingleQuots components except for VEGF, bFGF, and EGF. Subsequently, the harvested cells were resuspended in the same medium (1×10^3 cells/mL) and were cultured under an atmosphere containing 5% CO_2 at 37°C for the prescribed number of days.

Surface coverage with cells was estimated by digital counting of phase-contrast micrographs of cultured cells as an indicator of cell growth. When the immobilized VEGF was blocked with anti-hVEGF antibody, the antibody was first added to the culture medium at 1 μg/mL. The cells were stained with Giemsa at room temperature for 10 min after fixation of cells with methanol at 4°C for 10 min.

RESULTS AND DISCUSSION

Photoimmobilization of VEGF

VEGF was mixed with the photoreactive gelatin, and the mixture solution was dropped onto a polystyrene plate and the plate was dried and photoirradiated. When the immobilized spot was stained using VEGF receptor, concentration-dependent binding of the receptor was observed, as shown in Figure 2. Thus the immobilized VEGF could actively interact with the cognate receptor. The active site of VEGF was therefore considered exposed to the medium. In addition, washing with ethanol did not significantly reduce the amount of bound receptors, so the immobilized VEGF was stable against ethanol.

Previously, we used azidophenyl-derivatized gelatin to immobilize erythropoietin and no release of immobilized erythropoietin was confirmed.[22] Therefore, in the present study, VEGF was considered to be quantitatively immobilized because the same method was employed for immobilization.

Cell culture

In the following experiments, HUVECs were cultured under starved conditions. The culture medium

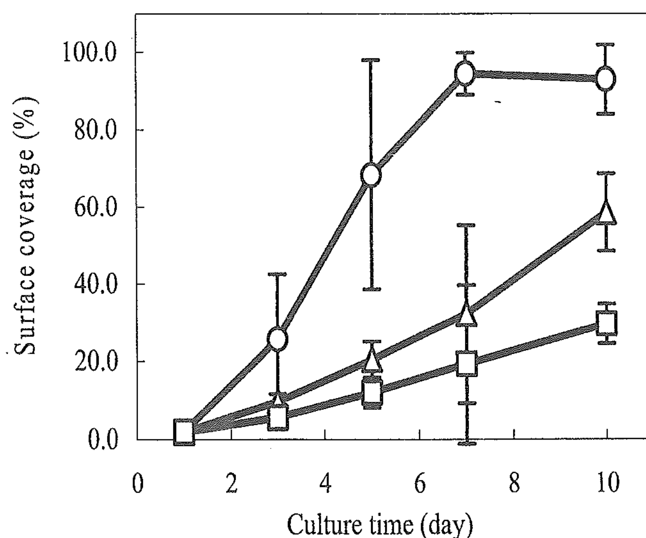

Figure 4. Cell growth of HUVECs on tissue culture polystyrene dishes immobilized with VEGF (□; 0.32, △; 3.2, and ○; 15.9 ng/mm^2). The surface concentration of gelatin was 15.9 ng/mm^2. Surface coverage by HUVEC was determined by phase contrast microscopy (n = 3).

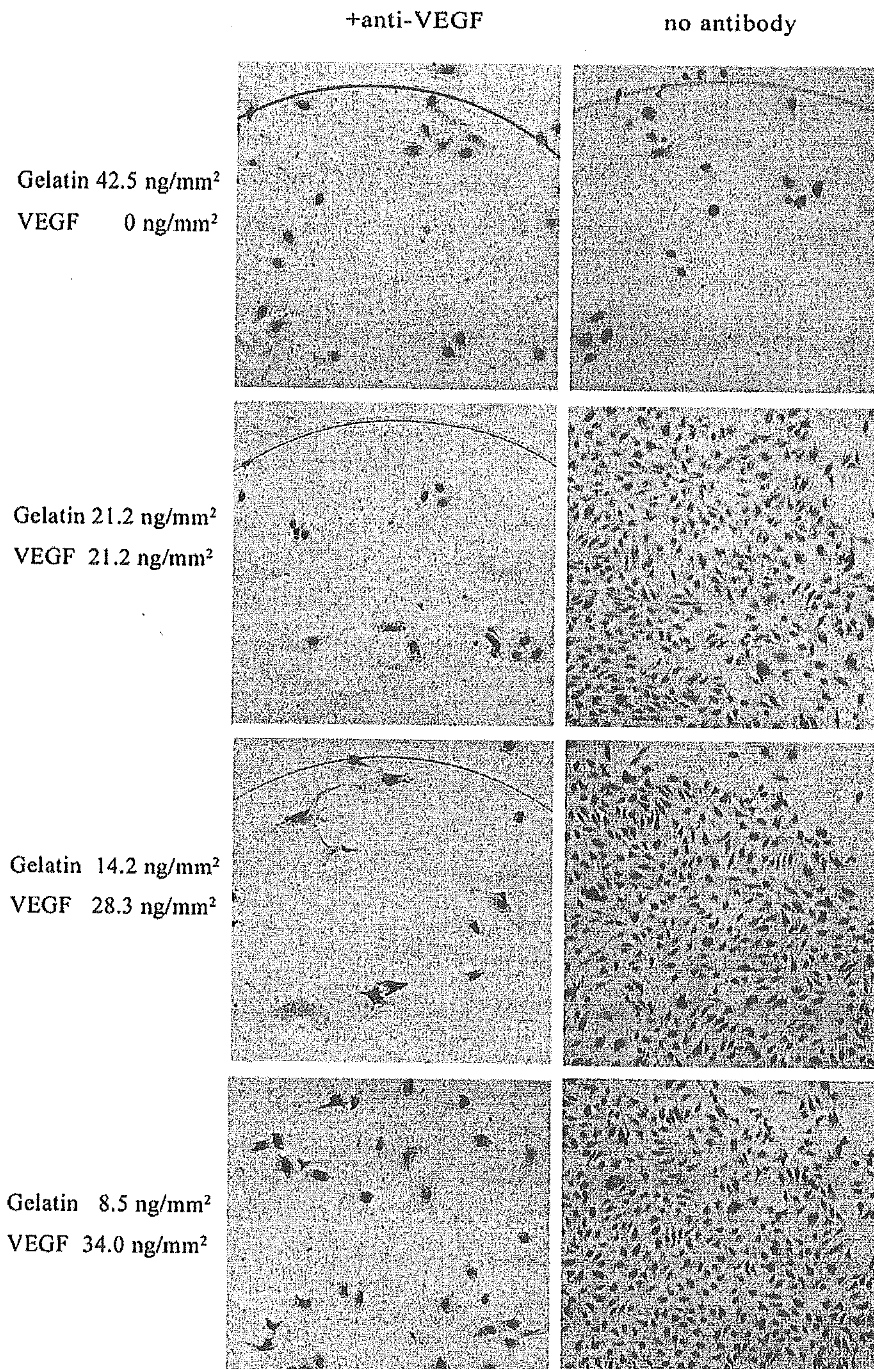

Figure 5. HUVEC growth on a tissue culture polystyrene dish immobilized with VEGF in the absence and the presence of anti-VEGF antibody. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

contained only 0.5% FCS and no angiogenic growth factors (bFGF, VEGF, or EGF). When HUVECs were placed on the microspot-immobilized VEGF surface and cultured, the cells adhered to the VEGF-immobilized regions as well as to nontreated regions (polystyrene surface). There was no significant increase in cell numbers after 1 day of culture. However, after 3 days, the numbers of cells on the VEGF-immobilized regions were significantly higher than on nontreated regions, as shown in Figure 3. Neither significant adhesion nor growth was observed on gelatin-immobilized regions lacking VEGF. These results indicated that no significant release of VEGF occurred to affect the HUVECs' behaviors.

Immobilized VEGF enhanced cell growth in a concentration-dependent manner as shown in Figure 4. Cell growth was linear until 7 days and the surface was completely covered with cells at a concentration of 15.9 ng/mm^2 immobilized VEGF. After 1 day, the cell concentration was 17.1 $\pm$ 2.9 cells/mm^2, which