Measuring Stability of Vascular Endothelial Growth Factor using an Immobilization Technique

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Abstract Although immobilization of growth factors (GFs) on culture substrates has been investigated as a cell culture technique, quantification of the bioactive stability of immobilized GFs has not been studied in detail. We developed a system of measuring GF stability using heparin-immobilized collagen substrate, vascular endothelial growth factor (VEGF), and human umbilical vein endothelial cells (HUVECs). VEGF solution was added to a heparin-crosslinked substrate and immobilized on the substrate. HUVECs were cultured on the VEGF-immobilized substrate, and the mitochondrial activity of the cells was assessed. A calibration curve showing the relation between HUVEC mitochondrial activity and immobilized VEGF density was constructed. Next, immobilized VEGF and VEGF solution were pre-incubated at 37°C, and the pre-incubated VEGF was added to heparin-crosslinked substrate. HUVEC mitochondrial activity declined as the pre-incubation period increased. Density of active immobilized VEGF was derived from HUVEC mitochondrial activity using the calibration curve, and the effect of pre-incubation of VEGF at 37°C was demonstrated quantitatively. Even after culture for 16 days, immobilized VEGF in culture medium at 37°C retained 43% of the initial bioactivity. Immobilized VEGF retained activity better than VEGF solution for at least 12 days of pre-incubation. The present results indicate that immobilization improves the stability of VEGF.

Keywords : vascular endothelial growth factor, immobilization, stability, quantitative evaluation, cell culture.

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1. Introduction

Cell culture is essential in biological research and development. Growth factors (GFs) are critical for the maintenance of organ-specific functions and proliferative activity of cells grown *in vitro*. GFs also mediate diverse physiological processes such as vascularization and liver regeneration [1–3]. Moreover, GFs function as signal transmitters between cells or between cells and extracellular matrix, by specifically binding to receptors of target cells [4]. When used in cell cultures, GFs are usually added to liquid media, but recent studies have evaluated the immobilization of GF on cell culture substrate and matrix [5, 6]. Some advantages anticipated from using immobilized GFs include the need for smaller quantities of GF, improved stability, and possible *in vivo* applications such as tissue engineering or drug delivery [7]. Various GF-immobilized substrata and matrices have been developed [8–13], and some animals experiments using immobilized GFs have been reported [14, 15].

Although the use of immobilized GFs clearly requires

quantitative evaluation of their stability, this aspect is barely reported. One investigation evaluated the stability of immobilized GF on storage at 4°C, but did not quantitatively confirm the stability of its bioactivity [11]. Very few details on the stability of immobilized GF under culture conditions are available.

We therefore developed an evaluation system to quantitate GF bioactive stability using vascular endothelial growth factor (VEGF) as a GF model in human umbilical vein endothelial cells (HUVECs) at the *in vivo* temperature of 37°C. HUVECs were selected because they form a homogeneous monolayer in 2D culture with little cytomorphologic influence, and have been shown to proliferate on VEGF-immobilized substrates [12].

2. Methods

2.1 Cell and culture media

HUVECs and HuMedia-EG2 were purchased from Kurabo (Osaka, Japan). HuMedia-EG2 contained the basal medium HuMedia-EB2 supplemented with 2% (v/v) fetal bovine serum (FBS), 1 µg/ml hydrocortisone, 50 µg/ml gentamicin, 50 ng/ml amphotericin B, 10 ng/ml human recombinant epidermal growth factor (EGF), 5 ng/ml human recombinant basic fibroblast growth factor (bFGF), and 10 µg/ml heparin. HuMedia-C-FBS-EG2 contained 2% (v/v) charcoal/dextran-treated FBS (C-FBS, Thermo Fisher Scientific, Kanagawa, Japan), 1 µg/ml hydrocortisone, 50 µg/ml gentamicin, and 50 ng/ml amphotericin B in HuMedia-EB2.

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2.2 Culture substrate

We prepared heparin-immobilized collagen film culture substrate. This substrate was confirmed by culturing primary hepatocytes using immobilized hepatocyte growth factor (HGF) [16]. In addition, the effect of GF immobilized on heparin-immobilized collagen gel has been confirmed in transplantation experiments [17, 18].

Cellmatrix Type I-C (3 mg/ml; Nitta Gelatin, Osaka, Japan) was used to prepare collagen film. We prepared a 0.3 mg/ml collagen solution and adjusted the pH to 3.0 by adding 0.1 N HCl. We dispensed 250 μ l of the collagen solution into each well of a 48-well plate (bottom area 1.1 cm²; Thermo Fisher Scientific, Kanagawa, Japan) and air-dried the plates on a clean bench for 1 day to obtain collagen films. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) solution was prepared by adding 8 mg/ml EDC (Peptide Institute Inc., Osaka, Japan) and 2 mg/ml NHS (Wako Pure Chemicals, Osaka, Japan) to 0.05 M 2-morpholinoethanesulfonic acid, monohydrate (MES, pH 5.6; Dojindo Laboratories, Kumamoto, Japan) buffer. Dried collagen films were washed with MES buffer. EDC/NHS solution (200 μ l) was added to each collagen film and left to crosslink for 4 h. Collagen films were then washed with MES buffer.

We next prepared heparin solution by adding 10 mg/ml heparin (Wako Pure Chemicals), 10 mg/ml EDC and 6 mg/ml NHS to 1 ml of 0.05 M MES buffer. This heparin solution was diluted 10-fold (1 mg/ml heparin) and 100-fold (0.1 mg/ml heparin) with MES buffer. The three concentrations of heparin were individually added to crosslinked collagen films and allowed to react for 2 h. The films were then washed with 0.1 M Na₂HPO₄ to stop the reaction.

The quantity of immobilized heparin was determined using toluidine blue (TB). After immobilization, the heparin solution in each well was collected and mixed with TB solution [0.01% (w/v) TB, 3.36 mg/ml citric acid, 23.4 mg/ml disodium hydrogenphosphate]. The mixture was mixed on an orbital shaker overnight at room temperature, resulting in complexation of TB with heparin. The TB/heparin complex was then separated by centrifugation at 6700 $\times$ g, and the absorbance of the supernatant solution was measured at 540 nm using a microplate auto colorimeter (Immuno Mini NJ-2300; Biotec Co., Tokyo, Japan). The quantity of non-immobilized heparin was calculated from a calibration curve obtained using various concentrations of heparin. The density of immobilized heparin was calculated by subtracting the non-immobilized heparin from total heparin.

2.3 VEGF immobilization

VEGF and the assay kit (Duo Set ELISA Development kit) were purchased from R & D Systems Inc. (Minneapolis, MN, USA). Ten percent C-FBS solution was prepared in calcium- and magnesium-free phosphate buffered saline (CMF-PBS). To inhibit adsorption of VEGF, films were blocked with C-FBS solution for 1 h. VEGF solutions at various concentrations were prepared in HuMedia-C-FBS-EG2. VEGF solution (200 μ l) was added to heparin-

immobilized collagen film (20.2 μ g/cm² heparin) and left to react for 3 h at room temperature. VEGF was immobilized on films because of high affinity between VEGF and heparin.

VEGF concentrations were measured using a Duo Set ELISA Development kit. The density of immobilized VEGF on film was estimated by assaying the concentration of unbound VEGF in solution and subtracting it from total VEGF concentration. The ratio of immobilized VEGF was calculated by dividing the quantity of immobilized VEGF by the initial quantity of VEGF.

2.4 Cell culture

HUVECs were seeded onto VEGF/heparin-immobilized collagen films at a density of 5.0×10^3 viable cells/cm², in 220 μ l of EGF- and bFGF-free HuMedia-EG2, and cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. For experiments, HUVECs at the second passage were used. Medium was changed to HuMedia-C-FBS-EG2 1 day after seeding and every 2 days thereafter.

2.5 Mitochondrial activity assay

As an index of viable cell number, mitochondrial activity of HUVECs was estimated by a water soluble tetrazolium salts-8 (WST-8) assay. Briefly, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt] produces a formazan dye on reduction by intramitochondrial dehydrogenase [19]. We diluted WST-8 solution (Cell Counting Kit-8, Dojindo Laboratories) 1 in 10 in HuMedia-EG2, and incubated HUVECs in this tetrazolium-containing medium for 4 h. After incubation, an equal volume of 1 N HCl was added to stop the reaction. Formazan concentration in the medium was obtained by measuring absorbance at 450 nm using a microplate autocolorimeter.

2.6 Stability of VEGF

VEGF solution at 40 ng/ml was prepared in HuMedia-C-FBS-EG2. To evaluate the stability of immobilized VEGF, we added 200 μ l of VEGF solution to heparin-immobilized collagen films (20.2 μ g/cm² heparin) after blocking, and left to immobilize for 3 h at room temperature. The VEGF solution was changed to HuMedia-C-FBS-EG2 and the VEGF/heparin-immobilized collagen films were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C (pre-incubation) for 6, 9, 12, or 16 days.

To evaluate the stability of VEGF in medium, VEGF solution was pre-incubated before immobilization for 6, 9, 12, 16, or 29 days. We added 200 μ l of the pre-incubated VEGF solution to heparin-immobilized collagen films after blocking and left to immobilize for 3 h at room temperature.

HUVECs (220 μ l) suspended at 5.0×10^3 viable cells/cm² in EGF- and bFGF-free HuMedia-EG2 were seeded onto the two types of VEGF/heparin-immobilized collagen films. The medium was changed to tetrazolium-containing medium at day 1, 3, or 5 after seeding, and HUVEC mitochondrial activity was evaluated by WST-8 assay.

2.7 Statistical analysis

The results are expressed as mean $\pm$ standard deviation. Statistical analysis was performed using a two-tailed unpaired Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Culture substrate

The TB assay confirmed immobilization of heparin on collagen film, and showed that the amount of immobilized heparin depended on the initial heparin concentration. The immobilized heparin density was $30 \mu\text{g}/\text{cm}^2$ when using $10 \text{ mg}/\text{ml}$ heparin (**Fig. 1A**). The immobilized efficiency of heparin was found to be 76% at $0.1 \text{ mg}/\text{ml}$ heparin, and decreased with increased heparin concentration (**Fig. 1B**). The decreased reaction efficiency was probably due to excessive heparin relative to the amino groups in collagen films [20].

3.2 VEGF immobilization

The results of ELISA indicated that the density of immobilized VEGF increased almost in direct proportion to the initial VEGF concentration (**Fig. 2**). The efficiency of VEGF immobilization was 90% at $40 \text{ ng}/\text{ml}$ VEGF, approximately $10 \times$ that of liquid medium. Because the molecular weight of heparin is $\leq 30 \text{ kDa}$, at least 4250

times more heparin than VEGF molecules were considered to be immobilized on the films.

3.3 Relationship between VEGF and cell growth

Mitochondrial activity of HUVECs cultured on films immobilized with VEGF at various densities increased over time for all the VEGF densities tested (**Fig. 3A**),

Fig. 2 Relation between density of VEGF immobilized on substrate and initial VEGF concentration.

Fig. 1 TB assay. (A) Density of immobilized heparin on collagen film substrate at various initial heparin concentrations. (B) Rate of heparin immobilization on collagen film substrate at various initial heparin concentrations; $n = 3$. Bars represent S. D. * $P < 0.01$, ** $P < 0.05$.

Fig. 3 HUVEC mitochondrial activity and density of immobilized VEGF. (A) Changes in mitochondrial activity over time at various densities of immobilized VEGF (■, $6.49 \text{ ng}/\text{cm}^2$; □, $3.13 \text{ ng}/\text{cm}^2$; ▲, $1.70 \text{ ng}/\text{cm}^2$; △, $0.84 \text{ ng}/\text{cm}^2$; ◆, $0.42 \text{ ng}/\text{cm}^2$; ◇, $0 \text{ ng}/\text{cm}^2$); $n = 3$. Bars represent S. D. (B) Relation between density of immobilized VEGF on substrate and HUVEC mitochondrial activity on the 5th day of culture.

indicating that HUVECs proliferated over time. The growth behavior of HUVECs cultured on immobilized VEGF at 12.8 ng/cm^2 was similar to that at VEGF density of 6.49 ng/cm^2 (data not shown). HUVEC mitochondrial activity on the 5th day of culture increased with increase in immobilized VEGF density (**Fig. 3B**), indicating that HUVEC proliferation depended on immobilized VEGF density. Almost the same trend was observed by optical microscopy, and a pavement-like monolayer appeared on the 5th day of culture (**Fig. 4**).

3.4 Stability of VEGF

First, films with immobilized VEGF at a density of 6.49 ng/cm^2 were pre-incubated for various periods at 37°C . When HUVECs were cultured on the pre-incubated immobilized-VEGF films, mitochondrial activity increased over time (**Fig. 5A**), whereas HUVEC mitochondrial activity on the 5th day of culture decreased gradually with increase in pre-incubation period of VEGF-immobilized films (**Fig. 5C**). These results indicated that immobilized VEGF was deactivated during pre-incubation at 37°C in a time-dependent manner.

Second, 40 ng/ml VEGF solution was pre-incubated at 37°C for various periods before VEGF-immobilization. When HUVECs were cultured on VEGF-immobilized films prepared with the pre-incubated VEGF solutions, mitochondrial activity increased for all the pre-incubation periods tested (**Fig. 5B**). HUVEC mitochondrial activity on the 5th day of culture declined with increase in pre-incubation period of VEGF solution (**Fig. 5C**). These results indicated that VEGF solution was deactivated during pre-incubation at 37°C , in a time-dependent

manner.

Next, we considered that **Fig. 3B** is a calibration curve showing the relation between HUVEC mitochondrial activity on the 5th day of culture and density of immobilized VEGF. Using this calibration curve, we converted the HUVEC mitochondrial activities on the 5th day of culture shown in **Fig. 5C** to densities of active immobilized VEGF. The resulting curves (**Fig. 6**) show quantitatively the effect of pre-incubation at 37°C on VEGF stability. Even after 16 days of pre-incubation at 37°C , 43% of the initial quantity of VEGF maintained its biological activity when immobilized. In addition, immobilized VEGF retained activity better than VEGF solution for at least 12 days of pre-incubation, which suggests that immobilization improves the stability of biological activity of VEGF.

4. Discussion

Immobilization of GFs is being actively studied for clinical applications such as wound healing and tissue engineering. Investigations of GF-immobilized matrices for vascularization, nerve regeneration and tissue regeneration, and in vivo application of immobilized GF in animal experiments have been reported [14, 15, 17, 18]. However, excess GF is often immobilized on the matrices without optimization, and there is little information on the deactivation of immobilized GF used in culture or in vivo. Excess GF not only increases the cost but may also be biologically harmful [21, 22]. To optimize the use of immobilized GF, we developed the present system to evaluate GF stability during the culture period using HUVEC, VEGF, and heparin-immobilized collagen sub-

Fig. 4 Phase-contrast microscopic observation of HUVECs. (A) Film immobilized with 0 ng/cm^2 VEGF on the 1st day of culture; (B) film immobilized with 0 ng/cm^2 VEGF on the 5th day of culture; (C) film immobilized with 6.49 ng/cm^2 VEGF on the 1st day of culture; (D) film immobilized with 6.49 ng/cm^2 VEGF on the 5th day of culture. Bars indicate $200 \mu\text{m}$.

Fig. 5 Effect of pre-incubation period at 37°C on HUVEC mitochondrial activity. (A) Changes in mitochondrial activity over time for immobilized VEGF pre-incubated for various periods (◆, 0 day; □, 6 days; ●, 9 days; △, 12 days; ■, 16 days). VEGF was immobilized on films and then pre-incubated for various periods at 37°C. HUVECs were cultured on these VEGF-immobilized films. (B) Changes in mitochondrial activity over time for VEGF solutions pre-incubated for various periods (◆, 0 day; □, 6 days; ●, 9 days; △, 12 days; □, 16 days; ◇, 29 days). VEGF solutions were pre-incubated at 37°C for the indicated times and then immobilized. HUVECs were cultured on VEGF-immobilized films prepared using the pre-incubated VEGF solutions. (C) Relation between pre-incubation period at 37°C and HUVEC mitochondrial activity on the 5th day of culture (●, pre-incubated immobilized VEGF; ○, pre-incubated VEGF solution); $n = 3$. Bars represent S. D.

Fig. 6 Stability of VEGF at 37°C (●, pre-incubated immobilized VEGF; ○, pre-incubated VEGF solution); $n = 3$. Bars represent S. D. * $P < 0.01$.

strate.

Initially, we confirmed immobilization of heparin to collagen films, and quantified the density of heparin immobilization (**Fig. 1**). VEGF was immobilized on this substrate at $\geq 90\%$ efficiency, and the density of immobilized VEGF increased almost in direct proportion to the increase in VEGF concentration (**Fig. 2**). HUVEC proliferation on VEGF-immobilized substrate depended on the density of immobilized VEGF, and HUVEC mitochondrial activity on the 5th day of culture increased with increase in immobilized VEGF density (**Fig. 3**). Our calibration curve shows the relationship between the density of bioactive immobilized VEGF at the start of culture and HUVEC mitochondrial activity on the 5th day of culture, thus allowing the evaluation of bioactivity of immobilized VEGF using HUVEC cultures (**Fig. 3B**).

To evaluate the stability of immobilized VEGF, we simulated cell culture conditions by pre-incubating the VEGF-immobilized substrate in medium at 37°C. Although the immobilized VEGF presumably contains both bioactive and deactivated molecules after pre-incubation, general assay systems such as ELISA cannot detect bioactivity and therefore bioactive molecules cannot be quantified. However, we were able to quantify bioactivity of immobilized VEGF by culturing HUVECs on VEGF-immobilized substrate after pre-incubation and using our calibration curve (**Fig. 3B**; **Fig. 6**). Although the stability of biological activity of immobilized GFs has been evaluated by measuring survival or proliferation rate of model cells[11], decreases in these rates are only proxies for deactivation of immobilized GFs; they do not indicate deactivation directly unless the quantity of GF is directly proportional to the proliferative activity or survival rate of the cells. Therefore, quantitation of the stability of immobilized GF is possible for the first time using the calibration curve as shown in **Fig. 3B**. Our results also help to optimize the density of immobilized VEGF. For example, as 43% of the initial amount of VEGF retains its biological activity after 16 days' pre-incubation, an immobilization density $\geq 15.1 \text{ ng/cm}^2$ is needed for the immobilized VEGF to remain active for that period. This

technique offers a means of obtaining very important knowledge about immobilized GFs.

Growth factors in solution added to heparin-immobilized collagen substrates will generally become immobilized. Therefore, VEGF can be retrieved from a pre-incubated VEGF-supplemented medium simply by adding it to this substrate. Furthermore, the stability of VEGF in medium can be quantitatively evaluated and directly compared with immobilized VEGF by culturing HUVECs and using our calibration curve (**Fig. 3B**). The bioactive stability of both immobilized GF and GF solution can thus be evaluated using a similar system.

Our study also found that immobilized VEGF retained bioactivity better than VEGF solution after 12 days of pre-incubation. As heparin reportedly needs plural sulfate groups to bind with protein [23], such multipoint bonding is expected to improve the stability of immobilized VEGF. The bioactivity of immobilized VEGF was lower than that of VEGF solution after 16 days of pre-incubation. Some immobilized VEGF might have been detached during the pre-incubation period.

We believe that this evaluation system can measure bioactive stability of various GFs by selecting cells that respond to the target GF, and should provide the necessary information for practical application of immobilized GFs.

5. Conclusion

Using the novel evaluation system that utilizes VEGF, heparin-immobilized collagen substrate and HUVECs, the bioactive stability of immobilized VEGF was quantitatively evaluated and directly compared with the stability of VEGF solution. We found that even after pre-incubation for 16 days at 37°C, 43% of the initial amount of immobilized VEGF retained its biological activity. Our results suggest that immobilization improves VEGF stability.

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