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Development of a Fluorescent Peptide for the Detection of Vascular Endothelial Growth Factor (VEGF)

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A closed circulatory system for the supply of oxygen and nutrients to all tissues in the body exists in vertebrates; this system develops in early embryogenesis through vasculogenesis (blood vessel formation from progenitor cells) and angiogenesis (extension of blood vessels from preexisting vascular structures).[1,2] Angiogenesis is related to the proliferation and metastasis of cancer cells, and studies on the molecular mechanisms underlying angiogenesis have been recently undertaken.[3,4] The signal transducers ephrin,[5] angiopoietin,[6] vascular endothelial growth factor (VEGF), and others are known to be related to angiogenesis. VEGF is a major regulator of angiogenesis, and it promotes the migration and proliferation of endothelial cells and the formation of new blood vessels from preexisting capillaries.[7,8] VEGF is a homodimeric glycoprotein that exists in four major isoforms: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$.[9] Biological responses to VEGF expression result from the binding of VEGF to two membrane-embedded receptors and the subsequent intracellular signaling induced by receptor activation.[10] Therefore, it is important to establish a technology for the easy, rapid, and highly sensitive detection of VEGF.

Fluorescence spectrometry is a conventional, highly sensitive, analytical method, and fluorescent probes that exhibit a spectral response upon binding with ions or neutral organic or inorganic molecules have enabled researchers to investigate the changes in free guest ions or in the concentrations of molecules by employing fluorescence microscopy, flow cytometry, and fluorescence spectroscopy.[11–13] Moreover, fluorescence-based assays for proteins, inhibitors, and probes can be advantageous in terms of high-throughput analysis if used not only in assay formats that involve an immobilized component, but also in the solution state.[14]

We considered the following requirements while designing a fluorescent reagent to detect VEGF: 1) production of weak to strong fluorescence upon binding to VEGF—this would potentially eliminate background noise and result in the highly sensitive detection of VEGF, 2) high selectivity and sensitivity, 3) reduced interference from foreign substances and 4) the ability to immobilize the fluorescent reagent onto a plate, and thereby increase the ease of handling.

In this study, we developed an excellent fluorometric reagent, referred to as **1**, by incorporating the above requirements (Figure 1). This reagent possesses a peptide as the binding site of VEGF, and the amino acid sequence of the peptide has a high affinity for the tyrosine kinase receptor kinase insert domain-containing receptor (KDR) or fms-like tyrosine kinase-1 (Flt-1) of VEGF.[15–17] The fluorophore fluorescein is introduced at the N terminus of the peptide through the isothiocyanate group. The lysine at the C terminus of the peptide has a role in immobilizing the peptide onto the plate.

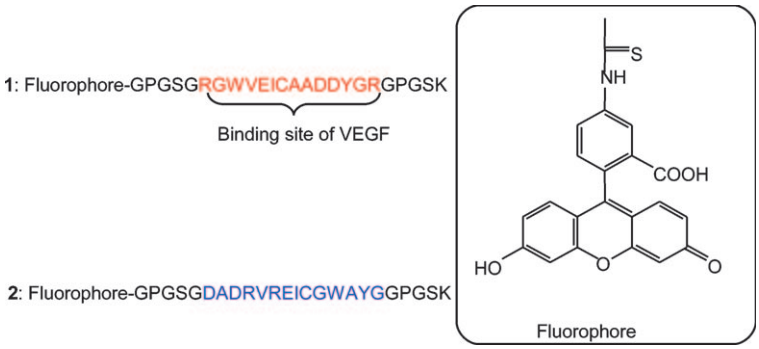

Figure 1. The chemical structure of the fluorescent VEGF probe (**1**) and the reference compound (**2**).

To study the in vitro photophysical properties of **1**, we recorded its fluorescence spectrum in a buffer (pH 7.0) at 25 °C. Figure 2A shows the fluorescence spectral changes in **1** before and after the addition of VEGF at varying concentrations, and Figure 2B shows a photograph of a solution of **1** with and without VEGF. Compound **1** by itself exhibited very weak emission, whereas the **1**–VEGF complex exhibited a strong green emission, with a dramatic increase in the fluorescence intensity centered at approximately 525 nm. The fluorescence spectral changes and strong emission occurred due to the formation of a complex in which the fluorophore in **1** bound to the hydrophobic residues of VEGF. The difference between the ground and the excited states is influenced by factors in the external environment of the fluorophore such as solvent polarity and desolvation. As a result, there was a linear change in the fluorescence intensity in response to the amount of VEGF in the solution.

Compound **1**, however, did not exhibit strong fluorescence in the presence of excess bovine serum albumin (BSA, 1.0 mg mL^{-1}; data are shown in the Supporting Information); this indicates that **1** has a high selectivity for VEGF.

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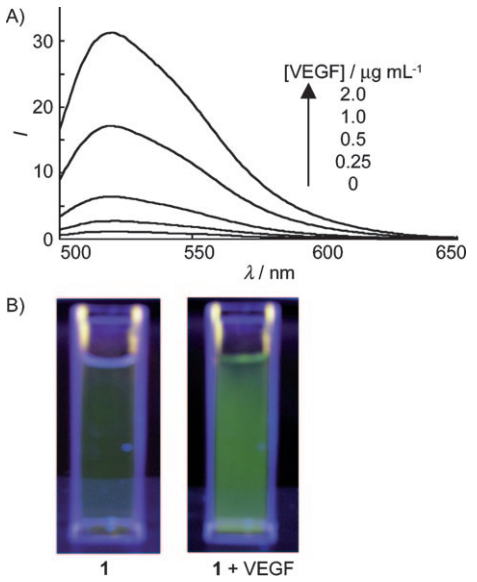

Figure 2. The fluorescent properties of **1**. A) The fluorescence spectra of **1** before and after the addition of 0–2.0 μg mL^{-1} VEGF and B) the photograph of the solution of **1** with and without 1.0 μg mL^{-1} of VEGF. Compound **1** = 10.0 μM in PBS (10 mM, pH 7.4). Excitation wavelength: 480 nm.

The emission intensities of **1** at 525 nm were plotted as a function of the VEGF concentration, and a typical calibration graph of the relation of the intensities and the VEGF concentration under optimum experimental conditions was obtained (Figure 3). This plot exhibited a good linear relationship between the emission intensity of **1** and the VEGF concentration ($r^2 > 0.984$) up to 2.0 μg mL^{-1}. The VEGF detection limit was 5.0 ng mL^{-1} (signal-to-noise ratio of 3.0), which is sufficient for clinical use.

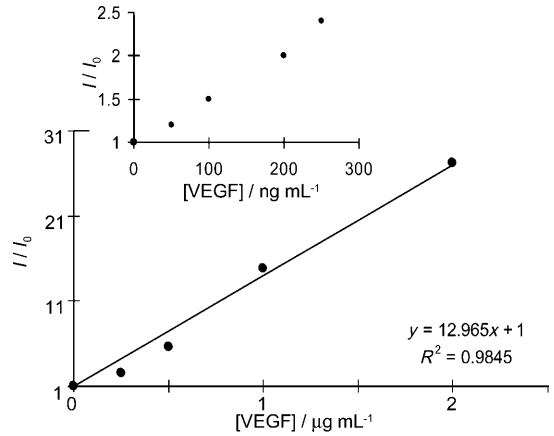

Figure 3. Plot of the fluorescence intensity at 525 nm as a function of the VEGF concentration. Compound **1** = 10.0 μM in PBS (10 mM, pH 7.4). Excitation wavelength: 480 nm.

To examine whether the amino acid sequence of the VEGF binding site in **1** contributes to the highly selective and sensitive detection of VEGF or not, **2** was synthesized as a reference compound (Figure 1). The VEGF binding site of **2** has the same amino acid composition as that of **1**, whereas the amino acid sequence of **2** differed from that of **1**. The fluorescence spectra of both **1** and **2** were monitored in the presence and absence of VEGF. The data are shown in the Supporting Information. Compound **2** exhibited a small change in the fluorescence intensity in the presence of VEGF (1.0 μg mL^{-1}). On the other hand, a remarkable change was observed in the fluorescence spectra of **1** after the addition of the same concentration of VEGF. These findings indicate that the amino acid sequence of the VEGF binding site in **1** played an important role in the increased fluorescence intensity and in the highly sensitive detection of VEGF.

The effects of various contaminants on **1** were examined in order to investigate the effect of these nonprotein substances on the accurate determination of VEGF (1.0 μg mL^{-1}). All the tests were carried out with a mixture of VEGF (1.0 μg mL^{-1}) and **1** (10.0 μM) in the presence of an excess amount of foreign substances. The details of the foreign substances used and the maximum concentrations that produce a fluorescence intensity perturbation of $< 10\%$ are summarized in Table S1. The results indicated that the response of **1** to VEGF was unaffected by the excess amount of nonprotein substances due to the formation of a stable **1**–VEGF complex.

To demonstrate the applications of **1**, it was immobilized onto an Au plate that was coated with self-assembled monolayers (SAMs), and the interaction between **1** and VEGF was monitored by surface plasmon resonance (SPR) and fluorescence spectrometry. The SAMs on the Au plate were derived by the reaction between 11-mercaptoundecanol triethyleneglycol ether and 20-(11-mercaptoundecanyloxy)-3,6,9,12,15,18-hexaoxaeicosanoic acid under optimum conditions.[18] Compound **1** was immobilized onto the mixed SAM by bond formation between the lysine in **1** and the carboxyl group in the SAM mediated by using *N*-hydroxysuccinimide (NHS) and *N*,*N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (EDC). After the coupling reaction, the residual activated NHS esters were quenched by the addition of an excess amount of ethanolamine. The immobilization procedure was monitored by the Biacore T100 system, and the data are shown in Figure S3 A. On the basis of the data, the amount of **1** immobilized on the plate was calculated to be 3.1 ng mm^{-2}. Figure S3 B shows the typical SPR sensorgrams of the immobilized **1** before and after the addition of various concentrations of VEGF. The SPR response of **1** increased as a function of the VEGF concentration. The K_d value between **1** and VEGF calculated from Figure S3 B was 2.0×10^{-7} M. These results indicated that immobilized **1** successfully interacted with VEGF. The fluorescence emission intensities of immobilized **1** at 525 nm were monitored after the addition of various concentrations of VEGF in phosphate-buffered saline (PBS, Figure 4). The fluorescence intensity of immobilized **1** increased as a function of the VEGF concentration, and a good linear relationship was observed. This phenomenon was consistent with the result obtained for the reaction of **1** with VEGF in the liquid phase. Further, the reaction of immobilized **1** with VEGF in rat serum was monitored by fluorescence spectrophotometry. A rapid increase in the fluorescence intensity of immobilized **1** was observed as a

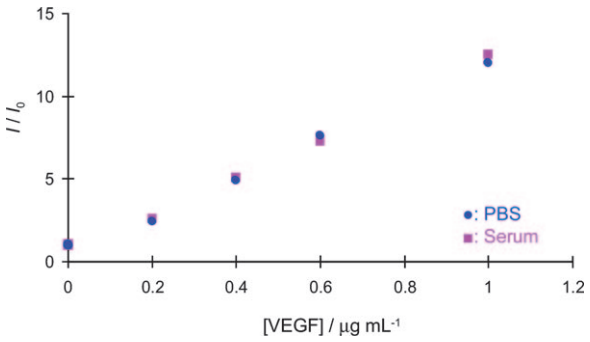

Figure 4. The plot of the fluorescence intensity of the immobilized **1** at 525 nm as a function of the VEGF concentration. Excitation wavelength: 488 nm. VEGF = 0–1.0 μg mL^{-1} in PBS (●) and in rat serum (■).

function of VEGF in rat serum at room temperature (Figure 4). This result indicated that the presence of foreign substances such as BSA and inorganic salts did not interfere with the reaction between immobilized **1** and VEGF.

Enzyme-linked immunosorbent assays (ELISAs) are widely used for VEGF detection. However, the use of ELISA for the detection of VEGF has the following disadvantages: 1) long time to obtain the results, 2) complex operation method, 3) difficulties in the reuse of the analytical reagent, 4) circulating VEGF could bind to serum proteins and become unavailable to ELISA antibodies and 5) incorrect results could be obtained due to the release of VEGF from platelets. Although our method is not as sensitive as the ELISA, the operation time for our method (1 min) is considerably shorter in comparison (ELISA takes 3 h); furthermore, our method allowed for the successful detection of VEGF in serum. Thus, the VEGF assay with **1** satisfies the requirements for the high-throughput analysis of VEGF, and it might prove to be widely applicable as a convenient method for VEGF detection in various scientific and medical fields.

The present study demonstrates a new, VEGF-binding, fluorescent peptide that interacted noncovalently with VEGF and exhibited a large increase in fluorescence intensity in response to VEGF binding. This peptide had a high selectivity for VEGF, and there was no interference in the detection of the proteins from the presence of foreign substances. In addition, the detection of VEGF in rat serum was successfully performed using the immobilized **1**. Recently, there has been emphasis on the early detection and treatment of cancer cells, and rapid progress has been made in angiogenesis research. This monitoring system with the VEGF indicator allows for the specific, easy and rapid detection of VEGF, with a low operating cost; therefore, it is a convenient method that can be widely applied for VEGF monitoring in hospitals, homes, and other fields.

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