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RESEARCH ARTICLE

Development of potency assays for a plasmid containing vascular endothelial growth factor 2

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Abbreviations: BrdU: 5-bromo-2'deoxyuridine
BSA: bovine serum albumin
CHO: Chinese hamster ovary
HLMVEC: human lymph microvascular endothelial cells
PBS: phosphate buffered saline
RSD: relative standard deviation
VEGF: vascular endothelial growth factor
VEGFR: vascular endothelial growth factor receptor

We have developed analytical methods to measure the biological functions of pVGI.1(VEGF2), a naked plasmid DNA product containing vascular endothelial growth factor 2 used in clinical trials for coronary artery diseases (CAD) and peripheral artery diseases (PAD). After being injected into muscles, vascular endothelial growth factor 2 (VEGF-2), presumably expressed in muscle tissues, binds to the endothelial cell receptors VEGFR2 or VEGFR3, triggering the downstream responses including cell proliferation and vascularization. As it is important to make sure clinical material is biological active, we developed a quantitative assay first to measure the receptor binding activity of the pVGI.1(VEGF2) gene product expressed by the transfected host cells, and then a qualitative assay to confirm the cell proliferation promoting activity of the expressed protein. In both assays the signals were plotted directly against input DNA concentrations used to transfect the host cells. We confirmed specificity for both assays. In addition, we demonstrated acceptable levels of spike recovery (86.7-116%), precision (largest relative standard deviation (RSD)=19.3%), linearity and range (60-140% relative potency, 15 - 35 µg/mL) for the quantitative assay. We intend to use the potency assays for routine lot release and stability studies.

The vector pVGI.1(VEGF2) is a 5283 base pair plasmid encoding the full length cDNA of the VEGF-2 (VEGF-C) gene. Preclinical studies and early phase clinical trials have demonstrated that injecting the pVGI.1(VEGF2) directly into muscles provided therapeutic angiogenic effects both in the heart and in the limb (Witzenbichler et al. 1998; Vale et al. 2001; Losordo et al. 2002; Fortuin et al. 2003; Kawamoto et al. 2004; Losordo and Dimmeler, 2004; Reilly et al. 2005; Shah and Losordo, 2005; Shintani et al. 2006). It was postulated that the pVGI.1(VEGF2) gene product, the secreted VEGF-2 protein, mediated proliferative and chemotactic responses in endothelial cells (Cao et al. 1998; Cao et al. 2004; Bauer et al. 2005), which resulted in formations of new blood vessels in the ischemic tissue.

The VEGF-2 protein is synthesized as a pre-propeptide that undergoes post-translational modifications including proteolysis and dimerization (Joukov et al. 1997). The mature VEGF-2 protein is a ligand for the vascular

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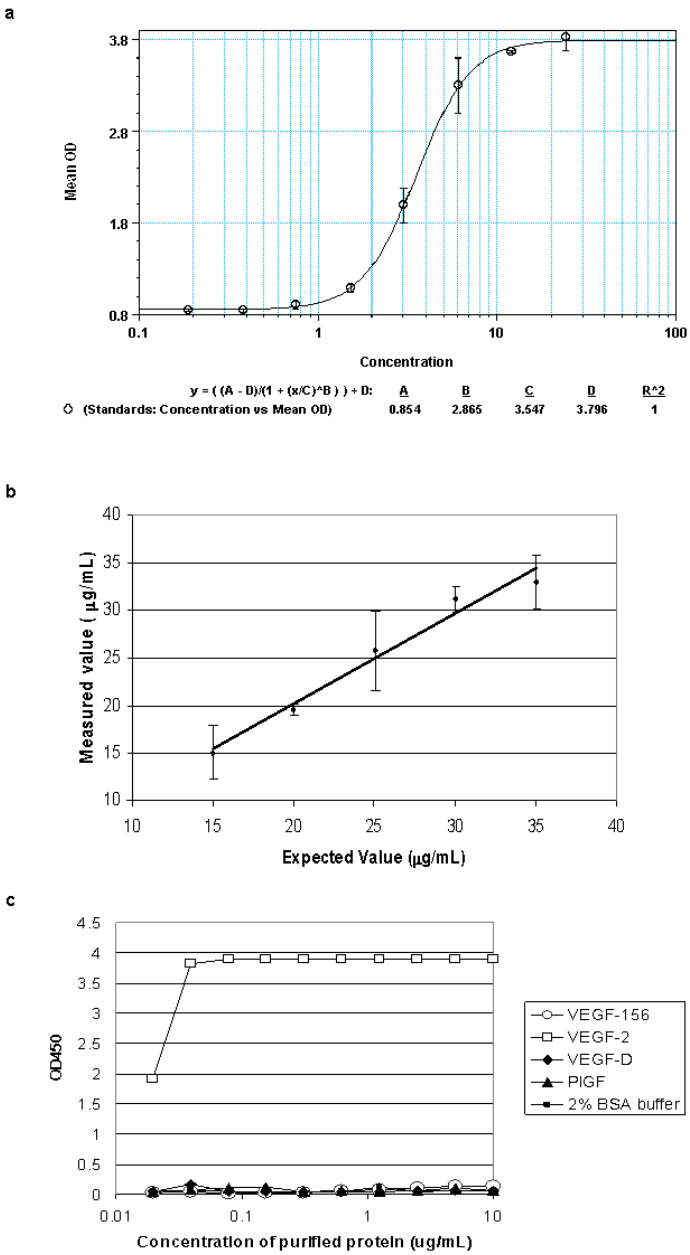

Figure 1. Quantitative receptor binding assay.

(a) Typical dose response curve for the quantitative potency assay. The pVGI.1(VEGF2) reference standard was pre-diluted to the starting concentration of 25 mg/mL before the 1:1 serial dilutions with the formulation buffer. The VEGF-2 in the conditioned media of the transfectants was then assayed for VEGFR3 binding activities (A450). The average of triplicate raw OD results was then plotted against the input DNA concentration in the transfection mix. The standard curve was modeled using a 4 parameter fit by SoftMax Pro v.3.1.

(b) The expected potency value vs. the measured potency value for the concentration range of 60-140% with the regression line (y = 0.949 x + 1.1617).

(c) Specificity of the binding reaction. The receptor binding detection was specific to the VEGF-C protein. Serially diluted protein was loaded on the plate and the binding signals were detected by OD_{450}. The background was assessed using BSA buffer that did not contain any protein from the VEGF family.

endothelial growth factor receptor (VEGFR)-2 and VEGFR-3, however, all intermediate forms of VEGF-2 binds to VEGFR-3 (Joukov et al. 1996; Jussila and Alitalo, 2002). The bound receptors then undergo auto-phosphorylation reactions that trigger downstream signal transduction cascades (Lee et al. 1996; Olofsson et al. 1999; Veikkola and Alitalo, 1999; Hamada et al. 2000; Veikkola et al. 2000; Makinen et al. 2001; Dixelius et al. 2003; Yong et al. 2005; Shibuya and Claesson-Welsh, 2006). The receptor binding is considered a first step for the pVGI.1(VEGF2) gene product to elicit its subsequent biological functions (Tomanek et al. 2002), which includes endothelial cell proliferation, a key process of angiogenesis (Tille et al. 2001; Persaud et al. 2004).

The objective of the work is to develop suitable potency assays for manufacturing lot release of pVGI.1(VEGF2). Ideally the potency assay must be able to measure clinically relevant biological function(s) of the gene product in a manner that fulfils the regulatory requirements in precision, accuracy, linearity and range (U.S. Food and Drug Administration, 2008). To achieve the goals we undertook the matrix approach by developing a combination of assays where the combined results constitute an acceptable potency assay. The potency assays consist of two methods: the quantitative relative potency assay, which utilizes receptor binding activity of the pVGI.1(VEGF2) gene product as the readout; and the qualitative cell proliferation assay, which confirms the downstream biological function of the expressed VEGF-2 protein by detecting the endothelial cell proliferation promoted by the protein secreted by the pVGI.1(VEGF2) transfected cells.

Here we report the development of both quantitative and qualitative potency assays to support pVGI.1(VEGF2) product release. The characterizations of the assays, including specificity of the qualitative assay, and accuracy, precision, linearity and specificity of the quantitative assay are also described.

MATERIALS AND METHODS

Plasmid DNA (pVGI.1(VEGF2)) and pVGI.0 preparations

The plasmid pVGI.1(VEGF2) contained the cDNA of human vascular endothelial growth factor 2 (VEGF-2, also known as VEGF-C) under the direction of a mammalian viral promoter and enhancer. The DNA used in assay development was produced under GMP conditions in various preparations. As negative control plasmid, pVGI.0 had the same plasmid backbone as pVGI.1(VEGF2) but without the VEGF-2 cDNA insert. Both plasmids were purified to high homogeneity (> 90% supercoiled form).

Cell lines and cell culture reagents

The Chinese hamster ovary (CHO)-K1 cell line was purchased from american type culture collection (ATCC) (Catalog number CCL-61) and maintained in RPMI1640 with 10% Fetal Bovine Serum. The 293H cell line was purchased from Invitrogen (Catalog number 11668-019 San Carlsbad, CA), and cultured in DMEM with 10% Fetal Bovine Serum. The primary human lymph microvascular endothelial cells (HLMVEC) were purchased from Cambrex (Catalog number CC-2812 Walkersville, MD), and cultured in EGM-2MV (Cambrex). CHO-5, the reduced serum medium for CHO-K1 cells, was purchased from Kemp Biotechonologies (Catalog number S0185 Frederick, MD) under a license agreement.

Transfection method for receptor binding assay

The CHOK-1 cells were first plated out in 96 well plates at a density of 5 X 10^4 cells/well. Both plasmid DNA (pVGI.1(VEGF2)) reference standard and test articles were first diluted to 25 µg/ml and then serially diluted with equal amounts of formulation buffer in 96 well dilution plates. The dilutions were made in triplicates. The transfection mix was prepared following the same plate format on a second 96 well plate. For each well, 50 µL of the diluted DNA was mixed with an equal volume of 25 µL/ml lipofectamine-2000™ in Opti-MEM-I®. The mix was incubated at room temperature for 30 min before the addition of 0.1 ml per well of CHO-5 medium. Immediately before transfection, the growth medium in the CHO-K1 cell plate was completely removed, and then 0.175 ml of the DNA/lipofectamine-2000™/CHO-5 medium was added to each well to start the transfection process. The transfection plates were incubated at 37ºC for 4 hrs. For gene expression of the transfected cells, the transfection medium was replaced with 0.15 ml/well of CHO-5 with 2 mM Glutamax (Invitrogen, Carlsbad, CA), 20 µg/ml recombinant human insulin (Sigma, St. Louis, MO), 0.2% FBS and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). The plates were then returned to 37ºC incubator with 5% CO_2 for 24-48 hrs before the receptor binding assay.

Transfection method for cell proliferation detection

The 293H cells were seeded in 24 well plates at a density of 5 X 10^5 cells/well a day prior to transfection. Plasmid DNA was serially diluted in Opti-MEM I (Invitrogen, Carlsbad, CA) and then mixed with an equal volume of 1:10 diluted lipofectamine-2000. The DNA:lipofectamine complex was incubated at room temperature for 20 min. Just before the end of the incubation, the medium from every well containing 293H cells was replaced with 0.35 ml of OptiMEM-I. The DNA:lipofectamine-2000 complex was then added to the 293H wells containing the Opti-MEM-I medium. The plates were incubated at 37ºC with 5% CO_2 for 4 hrs. At the end of the 4 hrs incubation period, 0.35 ml of DMEM with 20% FBS (Invitrogen) was added into each well. The plates were then further incubated at 37ºC with 5% CO_2 for 24 hrs.

Proliferation detection

The proliferation of the endothelial cells was detected using 5-bromo-2'deoxyuridine (BrdU) cell proliferation kit (CalBiochem Catalog number QIA48-1000) according to the instruction of the manufacturer.

Receptor binding ELISA

High binding ELISA plates (NUNC) were coated with 0.625 µg/ml recombinant human VEGFR-2(Flt-4)/Fc (R and D systems, Minneapolis, MN) at 4ºC overnight. The plates were then blocked with 2% bovine serum albumin (BSA) (Sigma, St. Louis, MO) in phosphate buffered saline (PBS) for 2 hrs at room temperature. Test samples were loaded into plates at 0.1 ml/well and incubated at room temperature for 2 hrs. The plates were washed three times

Table 1. Quantitative potency assay for pVGI.1(VEGF2) produced in different hosts and stability testing.

Experiment	Host	Treatment	DNA Concentration by A260 (mg/ml)	Potency Value Calculated by SoftMax Pro® (mg/ml)	Change in Response Curve (4 PL Parameters)
1	DH5a	Untreated	33	30	-
	DH5a	Heat treated (90ºC for 8 hrs)	33	9	Reduction in upper asymptote (ratio of standard/sample changed from 1 to 1.3)
	DH5a	Sheared (22 gauge needle for 30 times)	33	11	Reduction in upper asymptote (ratio of standard/sample changed from 1 to 1.3))
2	DH5a	Untreated	1000	1000	-
	Non-DH5a host	Untreated	1000	780	Change in parameter B (change from 3.383 ± 1.066 to 17.521)

with 0.3 ml/well of washing buffer, which was PBS with 0.05% Tween-20. The binding of VEGF-2 (VEGF-C) to the coated receptor was detected with biotinylated anti VEGF-C antibody (R and D systems, Minneapolis, MN) at room temperature for 2 hrs. After being washed three times with the washing buffer, the plates were stained with horse radish peroxidase (HRP) conjugated streptavidine (R and D systems, Minneapolis, MN) at room temperature for 20 mins. After another three washes with the washing buffer, 0.1 ml of the coloring reagent (R and D systems, Minneapolis, MN) was added into each well. The reaction was stopped with 0.1 ml/well of 1N HCl. The plates were read at A_{450} and A_{650} by Spectra Max 190 plate reader (Molecular Devices, Sunnyvale, CA).

Quantitative assay characterizations: accuracy and precision

Accuracy of the quantitative assay was evaluated by pre-diluting the reference standard at 60%, 80%, 100%, 120% and 140% of the test concentration for the assay and determining the recovery (%), which was defined as the following:

% Recovery=observed value/theoretical value x 100%

Precision was evaluated by the relative standard deviation (RSD) of at least three replicates.

Curve fitting and statistics

The dose response curves were modeled using a four-parameter logistic fit $Y=((A-D)/(1 + (x/C)^B)) + D$ by the SoftMax®Pro software (v.3.1 Standard, Molecular Devices). For reference standard, the graph was plotted using the A_{450} - A_{650} in the Y axis and DNA concentration in the X axis; for the test article (as an unknown dilution sample), the Y axis was also A_{450} - A_{650} but the X axis was the dilution factor. Other statistical analyses were performed using the GraphPad Instat Software (v.3.06, GraphPad Software, San Diego, CA) or Microsoft Excel spread sheet.

RESULTS AND DISCUSSION

Quantitative potency assay

The quantitative assay is based on the receptor binding activity of the pVGI.1(VEGF2) gene product. To simplify handling and minimize analyst errors, all operations, including serial dilutions, transfection and receptor binding detection were performed using a 96 well plate format. The transfection mix was prepared by mixing serially diluted pVGI.1(VEGF2) with a fixed amount of transfection reagent lipofectamine-2000™. The transfection mix was then transferred from the 96-well preparation plate to the corresponding location on the 96-well tissue culture plate containing the host cells to initiate transfection. The transfected cells were incubated for 24-48 hrs to allow sufficient time for VEGF-2 expression. The conditioned medium from each well was then collected and transferred to the corresponding location on the 96-well ELISA plate coated with a soluble VEGFR-3 receptor. The binding of the secreted VEGF-2 protein to the ELISA plate was detected by biotinylated anti-VEGF-2 antibody, and subsequent wash and incubation steps with horseradish

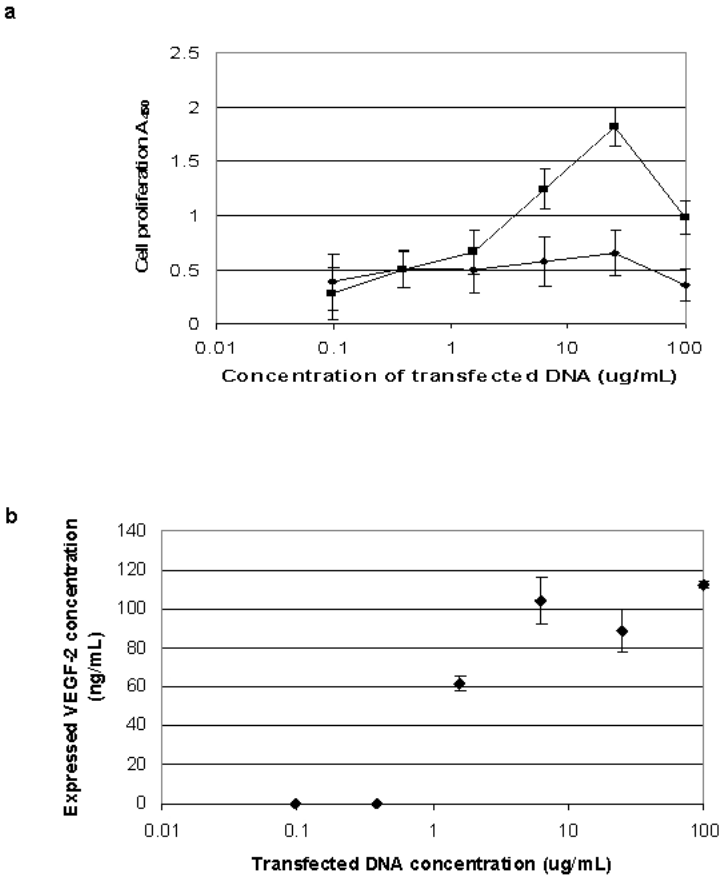

Figure 2. Qualitative cell proliferation assay.
(a) Endoethelial cell proliferation induced by supernatants of host cells transfected with serially diluted pVGI.1(VEGF2) (Squares) or the empty vector pVGI.0 (diamonds). Cell proliferation was detected by BrdU incorporation, followed by quantification of BrdU in the fixed and denatured cells. Each point is an average of 9 replicates. Error bars show the standard deviation of the 9 results.
(b) Quantification of the VEGF-2 protein expressed by serially diluted pVGI.1(VEGF2). The VEGF-2 protein was quantified by receptor binding assay, with purified VEGF-C protein (R and D systems) as standard. Each point is an average of 3 replicate. Standard deviation of the three is shown as the error bar.

peroxidase and coloring substrates. The raw data were analyzed by Softmax Pro® Version 3.0.

The A_{450} dose response curve (subtracted with the background noise by A_{650}) was a function of DNA concentration used in the transfection (Figure 1a). The coefficient of determination (R^2) was generally above 0.99 for the reference standard. The fitness of the curve was further examined by residual analysis, where the differences between the observed value and the theoretical value were plotted against the test concentrations. Visual inspection indicated that no obvious bias for any of the concentrations within the range (data not shown). The potency of unknown test article was then determined by comparing the curve of the unknown samples to that of the reference standard on the same plate without further data transformation.

The assay was characterized by accuracy and precision in the range of 60%-140% of the test concentration, which corresponded to 15-35 µg/mL of pVGI.1(VEGF2). The recovery ranged from 86.7% to 116% for 60%, 80%, 100%, 120% and 140% of target pVGI.1(VEGF2) concentration. The largest inter-day RSD was 19.3%. There was a linear correlation between the measured and expected values (Figure 1b) within this range with the coefficient of determination (R^2) of the regression line of 0.976. The linearity was further improved with a narrower range of 60-120% of the test concentration, for which the coefficient of determination (R^2) was 0.997. The result falls within the commonly-accepted range of about 25% RSD for cell based potency assays (Tuomela et al. 2005). Similar results have been obtained during assay validation after the assay was transferred to a QC laboratory (Waerner et al. 2007).

The assay was specific to the VEGF-2 expression of the pVGI.1(VEGF2) plasmid. When pVGI.0 was used for transfection, the receptor binding signals were not above the background (data not shown). The receptor binding detection system used for the potency assay was specific to

the VEGF-2 protein. Other proteins, such as VEGF-A, VEGF-D and PIGF from the VEGF family (Yancopoulos et al. 2000; Jussila and Alitalo, 2002; Tammela et al. 2005) had no detectable binding in the system (Figure 1c).

The assay was capable of distinguishing DNA isolated from a strain different from that of the reference standard (Table 1). The reference standard was isolated from *E. coli* host DH5α. The same plasmid DNA produced from a non-DH5α host generated a curve with the parameter B of 17.521, which was drastically different from the normal distribution of parameter B of reference standards (3.383 ± 1.066). Therefore, the response curve of the DNA from a new strain was considered non-parallel to the response curve of the reference standard. Furthermore, the reduced potency values were produced when heat damaged or fine needle sheered pVGI.1(VEGF2) was used for transfection (Table 1), suggesting the assay was stability indicative.

Qualitative potency assay

The qualitative biological activity assay was designed to confirm the biological function of the gene product of pVGI.1(VEGF2) as a complementary method of the quantitative potency assay. The principle of the assay was based on the cell proliferation promotion activity of the expressed VEGF-2 protein. Although many proliferation assays have been developed for purified proteins, it would be impractical to incorporate protein purification steps in the analytical method designed for qualifying plasmid DNA. An alternative strategy was to screen commercially available endothelial lines for their response to VEGF-2 protein secreted by the host cells transfected with pVGI.1(VEGF2) in conditioned media. The human lymph microvascular endothelial cells were selected based on the specific response to the VEGF-2 expression of the host cells.

As in the quantitative assay, serially diluted plasmid DNA in the transfection mix produced DNA concentration dependent proliferation responses. The procedure was similar to that of the quantitative assay. After 24 hrs of incubation, the supernatants of the transfected cells were transferred to 96 well plates containing HLMVEC (Kriehuber et al. 2001) after the removal of the host cells. The proliferation of HLMVEC was determined by BrdU incorporation followed by staining with anti-BrdU antibody provided by a commercially available BrdU cell proliferation kit (Calbiochem). The transfection step of the assay was performed in triplicate wells, and supernatants from each well were used to treat triplicate wells plated with HLMVEC. Therefore, at each dilution level, the proliferation results by the plasmid DNA were collected from optical density readings of nine wells. A triplicate mock transfection was included in each plate.

Figure 2a shows typical results of the qualitative potency assay. Proliferation was dependent on the presence of pVGI.1(VEGF2) in the transfection mix; and furthermore, the extent of proliferation was dependent on the concentration of pVGI.1(VEGF2), with an optimum at 1.25 µg/ml of pVGI.1(VEGF2). On the contrary, transfecting host cells with same amounts of pVGI.0 DNA did not result in supernatants that promoted proliferation. We have confirmed that the level of cell proliferation correlated with the level of VEGF-2 expression (Figure 2b) except for the highest DNA concentration tested, where the level of proliferation appeared to drop although the host continued to make large quantity of VEGF-2 protein.

Many factors affect the outcome of the qualitative potency assay. In addition to the age of the HLMVEC, the age of the host cell line, the lot to lot variation of fetal bovine serum used in the medium and the types of the media used to grow the host cells after transfection were all critical for the level of cell proliferation detected by the assay. These factors not only influence the quantity of the VEGF-2 protein expressed in the medium, but also the profile of all protein species present in the conditioned media. For example, during the initial screening exercise, the HLMVEC grown in DMEM based conditioned medium produced larger proliferation signal than those grown in MCDB131 based medium, even though MCDB131 was optimized to support clonal growth of human microvascular endothelial cells (Knedler and Ham, 1987). However, the VEGF-2 expression level, measured by VEGFR3 binding activity, was much lower in DMEM based medium than in MCDB131 based medium (data not shown). Further investigation is required to understand the roles of interactions between various components in conditioned media in promoting HLMVEC proliferation.

In conclusion, the potency assay methods, both qualitative and quantitative assays, fulfil the basic requirements for potency measurement for biological products. The characteristics of the combined assays, considering specificity, sensitivity, accuracy and precision, meet the requirements of product release and stability monitoring.

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