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Quantification of the vascular endothelial growth factor with a bioluminescence resonance energy transfer (BRET) based single molecule biosensor

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ABSTRACT

Neovascular pathologies in the eye like age-related macular degeneration (AMD), the diabetic retinopathie (DR), retinopathie of prematurity (ROP) or the retinal vein occlusion (RVO) are caused through a hypoxia induced upregulation of the vascular endothelial growth factor (VEGF). So far a correlation of intraocular VEGF concentrations to the impact of the pathologies is limited because of invasive sampling. Therefore, a minimally invasive, repeatable quantification of VEGF levels in the eye is needed to correlate the stage of VEGF induced pathologies as well as the efficacy of anti-VEGF treatment. Here we describe the development of three variants of enhanced BRET2 (eBRET2) based, single molecule biosensors by fusing a Renilla luciferase mutant with enhanced light output (RLuc8) to the N-terminus and a suitable eBRET2 acceptor fluorophore (GFP2) to the C-terminus of a VEGF binding domain, directly fused or separated with two different peptide linkers for the quantification of VEGF in vitro. The VEGF binding domain consists of a single chain variable fragment (scFv) based on ranibizumab in which the light- and the heavy- F(ab) chains were connected with a peptide linker to generate one open reading frame (orf). All three variants generate measureable eBRET2 ratios by transferring energy from the luciferase donor to the GFP2 acceptor, whereas only the directly fused and the proline variant permit VEGF quantification. The directly fused biosensor variant allows the quantification of VEGF with higher sensitivity, compared to the widely used ELISA systems and a wide dynamic quantification range in vitro. Our system demonstrates not only an additional in vitro application on VEGF quantification but also a promising step towards an applicable biosensor in an implantable device able to quantify VEGF reliably after implantation in vivo.

1. Introduction

Vascular endothelial growth factor (VEGF) as a strong endothelial-cell specific mitogen is affects physiological angiogenesis and lymphangenesis as well as pathological neovascularisation (Goel et al., 2013). VEGF is an anti-parallel, homo-dimeric, heparin-binding glycoprotein activating different intracellular signaling pathways through extracellular binding to the receptor tyrosine kinases (RTKs) VEGF-receptor1 (FLT1), VEGF-receptor2 (FLK1 or KDR) and VEGF-receptor3 (FLT4) located on the surfaces of endothelial cells promoting proliferation, migration, survival and vascular permeability (Kowanetz et al., 2006; Ferrara, 2004). Neuropilin receptors 1 and 2 (NRP1&2), initially identified as neuronal class3 semaphorin receptors in the developing nervous system, but also located on endothelial cells, primarily function as co-receptors, due to a lack of a catalytic intra-cellular domain, stabilizing VEGF-receptor binding (Geretti et al., 2008). The VEGF family consists of at least six subgroups (VEGF-A to VEGF-E) and the placental growth factor (PlGF). The VEGF-A subgroup is alternatively spliced to generate more or less soluble isoforms termed based on the number of amino acids (e.g.: VEGF-A165, 121, 189) (Ferrara, 2002). *In situ* hybridization demonstrated that the majority of human tumors express VEGF, indicating an involvement in tumor angiogenesis (Ferrara, 2004). VEGF expression is regulated by various cytokines or is oxygen dependent. Local hypoxic conditions such as in age-related macular degeneration (AMD), diabetic retinopathy (DR) or retinopathy of prematurity (ROP) cause an upregulation of VEGF expression in the eye, which leads to an uncontrolled formation of new but immature retinal or choroidal blood vessels impairing visual function due to edema and tractional retinal detachment. The state of the art treatment paradigm of these pathologies is the repeated injection of anti-VEGF drugs like bevacizumab (Avastin®), ranibizumab (Lucentis®) or aflibercept (EYLEA®), blocking the VEGF receptor binding

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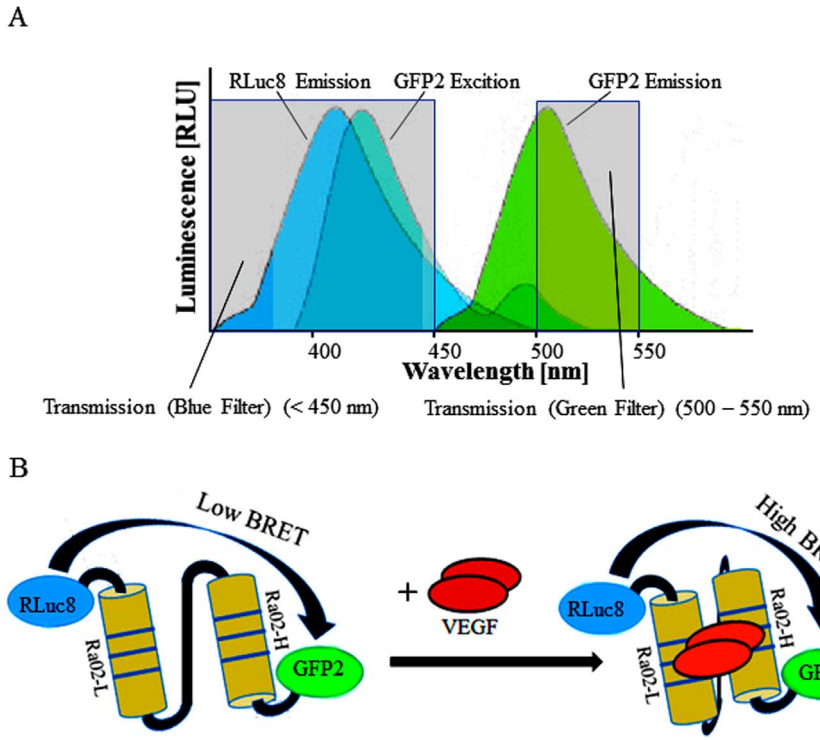

Fig. 1. eBRET2 principle. (A) RLuc8 converts coelenterazine 400a and thus generates an emission peak (~415 nm) overlapping the excitation peak of GFP2 transferring energy to the fluorophore which emits light at ~513 nm. (B) VEGF binding causes a conformational change in the biosensor by reducing the distance between RLuc8 and GFP2 and increasing the efficiency of the energy transfer which results in a higher BRET2 ratio.

domain of VEGF and thus inhibiting the VEGF induced VEGF receptor phosphorylation that leads to the activation of intracellular signaling pathways (Ferrara et al., 2004; Chen et al., 1999; Holash et al., 2002). The initial VEGF concentration before anti-VEGF treatment varies from patient to patient. Changes in visual acuity (VA) and morphologic alterations within the retina are measured with spectral domain optical coherence tomography (SD-OCT) to monitor the efficacy of the treatment occurring with delay and when VEGF inhibition is already decreasing again (Muether et al., 2012).

Biosensors offer a fast and sensitive confirmation and quantification of a wide spectrum of label-free analytes (Turner, 2013; Rea et al., 2011). Bioluminescence resonance energy transfer (BRET), a principle which uses a bioluminescent enzyme as a donor for the generation of energy that is transferred to a fluorescent acceptor molecule due to spectral overlapping, was already described by Theodore Förster in 1948 (Fig. 1A) (Foerster, 1948). This non-radiative energy transfer was extensively used to monitor and study protein-protein interactions in vitro and in living cells and tissues (Pfleger et al., 2006; De et al., 2007). Current BRET systems are mostly using the Renilla reniformis luciferase (RLuc) or related mutants with an increased light yield as the energy donor and variants of green fluorescent protein (GFP) with suitable spectral overlapping properties as the energy acceptor. RLuc´s emit light in the presence of coelenterazine derivates with different emission maxima (e.g.: $\lambda em = \sim 480$ nm for native coelenterazine (CLZ) or $\lambda em = \sim 400$ nm for coelenterazine400a (CLZ400a)) overlapping the excitation peak of GFP and thereby transferring energy to the fluorophore which emits light at a higher wavelength ($\lambda em = \sim 510$ nm). Because of the excellent spectral resolution ($\Delta\lambda em = \sim 100$ nm), the combination RLuc8 and GFP2, named after the mutational enhancement of the RLuc8´s light output and following the order of inventions (eBRET2), offers the most sensitive combination compared to other BRET systems (Loening et al., 2007). BRET systems are a useful laboratory tool not only to understand receptor function or protease activity, but also quantification of specific analytes was shown to be possible (Borroto-Escuela et al., 2013; Siddiqui et al., 2013; Wu et al., 2014; Dragulescu-Andrasi et al., 2011; Le et al., 2014).

The aim of this study was to develop protein-based, implantable biosensors for in vitro and in vivo VEGF quantification that reliably quantifies endogenous VEGF levels more sensitive than the commercially available ex vivo VEGF ELISAs (enzyme linked immunosorbent assays) (Fig. 1B). For this purpose we cloned the eBRET2 components RLuc8 and GFP2 to the N- and C-terminal part of a single chain variable fragment (scFv; Ra02) based on ranibizumab (Wimmer et al., 2015). This scFv was generated in our lab by fusing both chains together with a 6x glycine peptide linker to generate one open reading frame (orf). The directly fused biosensor molecule (RLuc8-Ra02-GFP2) was used to generate the two remaining biosensor variants RLuc8-Proline-Ra02-Proline-GFP2 and RLuc8–4xGlycine-Ra02–4xGlycine-GFP2 by PCR insertion mutagenesis (Supplementary Fig. 1).

2. Material and methods

2.1. Construction of eBRET2-VEGF-biosensors

Ranibizumab amino acid sequences (light- and antigen binding part of the heavy chain) were re-translated into DNA sequence, fused to the secretory IgG kappa leader sequence and codon optimized for the expression in mammalian cells. Both open reading frames (ORFs) were restriction cloned into pIREShrGFP (Stargene; La Jolla USA) separated with an internal ribosomal entry site (IRES). In the following step, the IRES and the heavy chain leader sequence were deleted again and a 6x glycine linker (GGC GGC GGC GGA GGA GGA) was introduced between the two ORFs, using mutagenesis polymerase chain reaction (PCR) to generate a VEGF binding single chain variable fragment (scFv) of ranibizumab