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The covalently immobilized antimicrobial peptide LL37 acts as a VEGF mimic and stimulates endothelial cell proliferation

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ABSTRACT

The chemical coupling of growth factors to solid substrates are discussed as an alternative to delivery systems. Utilizing entire proteins for this application is hampered by safety and stability considerations. Instead, growth factor mimicking peptides are of great interest for biomedical applications, such as tissue engineering, due to their purity and stability. The human cathelicidin derived antimicrobial peptide LL37, beside its microbicidal activity, was shown to stimulate endothelial cell growth when used in a soluble form. Here, in a novel approach, spacer mediated immobilization, but not direct conjugation of LL37, to a gold substrate was shown to result in a pronounced mitogenic effect on endothelial cells, comparable to that of soluble vascular endothelial growth factor.

1. Introduction

In tissue engineered (TE) scaffolds providing appropriate surface properties and the inclusion of growth factors (GFs) play a pivotal role in the initiation and maintenance of cellular growth and in directing differentiation. GFs find often application in release systems, where the GF is incorporated into the scaffold structure and released via diffusion into the environment to stimulate target cells.

An alternative approach makes use of ionically or covalently immobilized GFs that still exert their biological function, while coupled to a substrate surface [1]. One inherent advantage of this methodology is the immobilization itself that keeps the GF in the desired location and locally restricts its activity to the scaffold. Consequently, little or no systemic effects are to be expected. Nevertheless, utilizing whole proteins for this application is hampered by safety and stability considerations. Therefore, mimicking the biological function of entire proteins by small protein fragments is a common strategy in TE. Well known examples in this regard include peptides (mimotopes) that mimic the cell-adhesive function of extracellular matrix (ECM) proteins by providing specific adhesion motives, such as RGD, REDV, and others [2]. Thus, conjugation of such peptides to inherently non-adhesive materials enables cell adhesion and growth comparable to coatings with collagen, gelatin or fibronectin without undesirable effects frequently associated with such large biomolecules [3]. Furthermore, peptides comprise several advantages over full length proteins including well-defined synthesis, high purity, stability towards degradation, and sterility and all this at comparable low costs. Additionally, there is no risk in contamination with products of animal origin a crucial prerequisite for TE. Consequently, immobilization of GF-mimicking peptides on artificial substrates is highly appealing for TE. Yet, in this respect only few attempts have been described [4].

Vascular endothelial growth factor (VEGF) plays a central role in vascular development and in this respect, is of major importance for angiogenesis and the formation of new vessels inside engineered scaffold as well as for their colonization by cells [5]. The receptor-binding domain of VEGF consists of a 15 amino acid sequence, which in the context of the apoprotein forms an α-helical structure that binds and phosphorylates VEGF receptor 2 (VEGFR2) by forming a dimer. Without the peptide backbone, no interaction occurs. In order to “force” the 15-mer into an α-helix, appropriate modifications at both the N- and at the C-terminal end proved to be applicable [4]. This peptide was used for surface modifications in

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subsequent experiments [6,7]. However, these attempts showed only moderate biological effects.

The human cathelicidin derived antimicrobial peptide (AMP) LL37 was first described as a component of the innate immune system [8], able to kill bacteria and fungi while not harming mammal cells at physiological concentrations. In addition, LL37 has been described to be involved in chemotaxis, endotoxin neutralization, virus inactivation, and angiogenesis [9]. The latter was tested for the soluble AMP [10]. The pro-angiogenic effects seem to be mediated indirectly via stimulation of VEGF signaling. Hence, LL37 represents an interesting alternative to the above-mentioned VEGF derived peptide. In addition, we propose that keeping the (putative) ligand away from the surface by a long, flexible, and hydrophilic spacer, such as polyethylene glycol (PEG), facilitates ligand-receptor interactions by enhancing accessibility.

The aim of the present study was to test covalently immobilized LL37 for its biological activity on endothelial cells (ECs). This was accomplished by using a conjugation scheme adopted from our previous work [11]. As a measurement model, the ECIS (Electric Cell-substrate Impedance Sensing) system was used for label-free monitoring of EC attachment and growth.

2. Materials and methods

2.1. Reagents

Cysteamine, bovine serum albumin (BSA), 3-Maleimidopropionic acid N-hydroxysuccinimide ester (NHS-Prop-Mal), gelatin and general chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human serum albumin (HSA) was purchased from Sanquin (Amsterdam, The Netherlands). Rapp-Polymere (Tübingen, Germany) provided α-N-hydroxysuccinimidyl-ω-maleimidyl-PEG (NHS-PEG-Mal, MW 3000 g/mol). Recombinant VEGF 165 was purchased from Immunotools (Friesoythe, Germany). Sulfosuccinimidyl 4-(4,4′-dimethoxytrityl) butyrate (sulfo-SDTB) was obtained from VWR (Radnor, PA, USA). The peptide LL37 extended by a N-terminal cysteine residue (C-LLGDFFR KSKEKIGKEF KRIVQRIKDF LRNLVPRTES, termed C-LL37) synthesized by standard Fmoc-chemistry was a kind gift provided by Kamran Nazmi (Oral Biochemistry, Amsterdam, The Netherlands). Chromium/gold-coated glass was a kind gift from Alex Lotz (Max Planck Institute for Polymer Research, Mainz, Germany).

2.2. Primary cell culture

Umbilical cords for the isolation of human umbilical vein endothelial cells (HUVEC) were collected at the Department of Obstetrics, Amstelland Hospital (Amstelveen, The Netherlands). The IRB of the VU University Medical Center (Amsterdam, The Netherlands) approved the work with HUVEC, written informed consent was provided, and none of the authors had access to any identifying information. HUVECs were isolated and cultured as described previously[13] using the method of Jaffe et al.[12]. In brief, a single cell suspension was collected from the umbilical vein after enzymatic tissue digestion with collagenase type II (6700 U/mL) and plated on 1% gelatin coated standard culture plates (Costar, Cambridge, MA, USA). The cells were maintained at 37 °C with 5% CO_2 in Earl's balanced M199 supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin (all Biowhittaker, Verviers, Belgium), 10% human serum (Sanquin, Amsterdam, The Netherlands), 10% new born calf serum (Gibco, Grand Island, NY), 5 U/mL heparin (Leo Pharmaceutical products, Weesp, The Netherlands) and 150 μg/mL ECGF. For the described experiments HUVECs of passage one were used.

2.3. Electric Cell-substrate Impedance Sensing (ECIS)

ECIS measurements to study cell attachment and growth in dependency on the different surface modifications were performed based on an extensive protocol published elsewhere [12]. ECIS arrays 8W10E PCB (Applied BioPhysics, Troy, NY, USA) were used. The gold-electrodes were first activated with cysteamine (18 mM in water) over-night. Alcohols and other organic solvents were tested and found to dissolve the insulation coating of the electrodes and thereby disable measurements. After rinsing, the surfaces were either incubated with 0.5 mg/ml NHS-Prop-Mal in phosphate buffer saline, pH 7.4 (PBS) containing 20% dimethylformamide or with 0.5 mg/mL NHS-PEG-Mal in PBS, respectively. The reaction was terminated after 3 h and samples were washed with water. Finally, the surfaces were treated with 0.5 mg/mL C-LL37 in 50 mM carbonate buffer, pH 9.0 for additional 3 h. After several washes with water the modified slides were air-dried and sterilized by exposure to UV-light for 20 min. Thereafter, HUVECs were seeded semi-confluent with a density of 20,000 cells/cm^2 in fully supplemented culture medium.

2.4. Attachment assay

Chromium/gold-coated glass slides with a surface area of 1 cm^2 were modified as described for the ECIS arrays. The amount of immobilized peptide was estimated using a previously described colorimetric assay using the compound sulfo-SDTB [11,13]. Thereafter, glass slides were sterilized with UV-light for 20 min and HUVECs were seeded confluent (60,000 cells/cm^2) directly on the slides without additional surface treatment. After 5 h incubation with fully supplemented culture medium, cells were washed two times with warm PBS and fixed with 2% paraformaldehyde in PBS for immunofluorescence staining.

2.5. Immunofluorescence staining

The F-actin cytoskeleton was stained with rhodamine-phalloidin (3 U/mL, Life Technologies, Carlsbad, CA, USA) and the nuclei with Hoechst 33258 (0.02 μg/mL, Sigma-Aldrich) following a previously published protocol [14]. The images were quantified with Slidebook version 5.5 (Intelligent Imaging Innovation, Denver, CO, USA). To this end, the number of attached cells was automatically determined by masking the nuclei from at least 10 images per condition.

2.6. Western blot

Antibodies purchased form Cell Signaling Technologies (Danvers, MA, USA) were used against phospho-VEGFR2 (2478, 1:500) and ERK1/2 (9102, 1:1000). Cells were grown to confluence on the surface-modified glass slides and lysates were prepared in the presence of a phosphatase and protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Risch, Switzerland).

2.7. Statistics

Number of biological replicas (n) is indicated. Data are represented as mean ± standard error of mean. Statistical differences were calculated using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) and p-values ≤ 0.05 were considered significantly different.

3. Results and discussion

Coupling of thiols to gold is the method of choice for this