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Short-range cytokine gradients to mimic paracrine cell interactions *in vitro*

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ABSTRACT

Cell fate decisions in many physiological processes, including embryogenesis, stem cell niche homeostasis and wound healing, are regulated by secretion of small signaling proteins, called cytokines, from source cells to their neighbors or into the environment. Concentration level and steepness of the resulting paracrine gradients elicit different cell responses, including proliferation, differentiation or chemotaxis. For an in-depth analysis of underlying mechanisms, *in vitro* models are required to mimic *in vivo* cytokine gradients. We set up a microparticle-based system to establish short-range cytokine gradients in a three-dimensional extracellular matrix context. To provide native binding sites for cytokines, agarose microparticles were functionalized with different glycosaminoglycans (GAG). After protein was loaded onto microparticles, its slow release was quantified by confocal microscopy and fluorescence correlation spectroscopy. Besides the model protein lysozyme, SDF-1 was used as a relevant chemokine for hematopoietic stem and progenitor cell (HSPC) chemotaxis. For both proteins we found gradients ranging up to 50 μm from the microparticle surface and concentrations in the order of nM to pM in dependence on loading concentration and affinity modulation by the GAG functionalization. Directed chemotactic migration of cells from a hematopoietic cell line (FDCPmix) and primary murine HSPC (Sca-1$^+$ CD150$^+$ CD48$^-$) toward the SDF-1-laden microparticles proved functional short-range gradients in a two-dimensional and three-dimensional setting over time periods of many hours. The approach has the potential to be applied to other cytokines mimicking paracrine cell–cell interactions *in vitro*.

1. Introduction

Cytokines and other small soluble mediator molecules play a crucial role in cell–cell communication of multicellular biological systems. Both long-range (>100 μm) and short-range gradients of these agents facilitate many cellular processes, including chemotaxis and differentiation during embryogenesis as well as in diseased or regenerating adult tissues. Short-range paracrine interactions between neighboring cells are based on secreted cytokines, which are diluted with increasing distance from the source cells, leading to the establishment of cytokine gradients. Many cellular processes are not sensitive solely to the presence of cytokines, but also to cytokine concentrations or gradients [1,2], so that cell fate decisions frequently depend on the distance between source and receiving cell. As already mentioned, one obvious example is pattern formation during embryogenesis, exemplified by the left–right symmetry created by reaction–diffusion pair Nodal–Lefty [3], or drosophila wing patterning by hedgehog (HH) [4] and decaplentaplegic (Dpp) [5]. However, paracrine signaling also plays a role during wound healing, where platelet-derived growth factor (PDGF) attracts fibroblasts [6] and transforming growth factor-β (TGFβ) induces their transformation into tissue-contracting myofibroblasts [7], as well as in tumor microenvironments [8] and in the regulation of stem cell niches [9]. In these microenvironments stem cell fate is balanced by a multitude of signals including paracrine cytokine gradients from neighboring cells [10,11]. Common examples of these functional entities are the intestinal crypt [12], the hair follicle [13], drosophila testes [14] and the hematopoietic stem cell (HSC) niche in the bone marrow [15,16].

A well-known phenomenon of cellular sensitivity toward cytokine gradients is directed migration of hematopoietic stem and progenitor cells (HSPC) toward gradients of stromal cell-derived factor-1 (SDF-1, also known as CXCL12). This directed movement under the influence of chemokines is referred to as chemotaxis. SDF-1 is a chemokine supporting the homing of HSPC into the bone marrow niche [17], where they show close localization to SDF-1-producing stromal cells

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[16,18]. SDF-1 acts *via* the G-protein coupled receptor (GPCR) CXC chemokine receptor type 4 (CXCR4) natively expressed on HSPC [19]. Recently the receptor CXC chemokine receptor type 7 (CXCR7) was identified as another target for SDF-1 [20]. It functions mainly as its scavenger thereby modulating cell's mobility [21].

In vivo the presentation, storage and gradient accumulation of many of those cytokines is facilitated by glycosaminoglycans (GAG) — an important component of the extracellular matrix (ECM) [22,23]. These polysaccharides are bound to proteins and besides hydration, their key function is the binding and local concentration of growth factors, cytokines and other mediators [23]. Different configurations and different degrees of sulfation determine cytokine binding, primarily *via* the variable density of negatively charged moieties. While hyaluronan (HA) is the only non-sulfated GAG, heparan sulfate and heparin belong to the most sulfated representatives. Because of this well-documented *in vivo* function the cytokine–GAG interaction has been already used for storage and release systems *in vitro* [24,25].

The high complexity of the *in vivo* tissue microenvironment and stem cell niches established by different cell types, cytokines and the ECM makes analyses and understanding of regulatory processes difficult. Hence, appropriate *in vitro* models are required to mimic physiological processes, including cytokine gradients [26]. Such *in vitro* models permit an in-depth analysis using a multitude of high-resolution techniques. In this way it is possible to get an enhanced understanding of the dynamic processes of disease and tissue development, often excelling static endpoint analysis obtained from *in vivo* experiments [27]. Additionally such biomimetic *in vitro* models can reduce the number of ethically controversial animal experiments. Several approaches exist for the formation of *in vitro* cytokine gradients. These include traditional methods like transwell assays (based on Boyden chamber) [28], which suffer the disadvantages of ill-defined gradients, endpoint analysis and whole cell population based read-outs. Other approaches use microfluidics to achieve mediator gradients, which can be precisely controlled and enable accurate prediction of a steady-state gradient [29]. However, these methods are frequently limited to long-range gradients over several hundred micrometers [30] and are not suited for mimicking short-range paracrine signals. Furthermore, they are technologically demanding and difficult to set up for multipole, complex spatial arrangements.

We aimed to set up a system to deliver short-range gradients of various mediators in different *in vitro* cell culture environments. The aim was to enable control over cytokine gradients at the cellular scale over several days to investigate cellular processes like migration, proliferation and differentiation. Moreover, the characteristics of the ECM microenvironment should also be controlled in order to better mimic physiological situations of stem cell niches, wound healing or tumor progression. We envisioned an arrangement as depicted in Fig. 1 with protein-laden microbeads (μ-beads) as surrogates for cytokine releasing cells and surrounding cells as the receiver of the paracrine signals of interest. Cells and μ-beads are embedded in suitable biomimetic ECM matrices, such as functional three-dimensional (3D) collagen networks as recently introduced [31–33]. Similar μ-beads have already been used as single point sources for proteins, based on either surface-bound [34] or soluble cytokines [6,35,36]. Exemplarily, such a setup should simulate the HSC bone marrow niche with the μ-beads acting as niche cell surrogates in a structured ECM environment similar to that occurring *in vivo*.

Our short-range gradient system was realized by the usage of cell-sized spherical agarose μ-beads with different GAG functionalization to control cytokine release. Uptake and release kinetics as well as gradient buildup were quantified from observing fluorescently labeled model proteins including lysozyme and SDF-1 using confocal laser scanning microscopy (cLSM) and fluorescence correlation spectroscopy (FCS). Finally, cell experiments using a hematopoietic cell line and primary murine hematopoietic stem and progenitor cells (HSPC) proved the presence of functional short-range cytokine gradients by chemotaxis toward SDF-1-laden μ-beads.

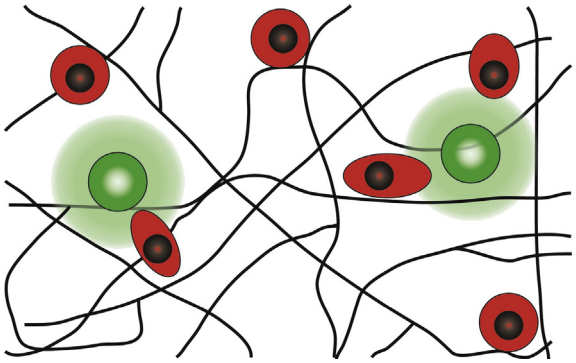

Fig. 1. Setup of short-range cytokine gradients from μ-beads to study paracrine cell signals. Protein-laden μ-beads (green) as a local protein delivery vehicle establish short-range, cell-sized protein gradients (pale green) in their proximity due to a slow protein release. Cells (red) and μ-beads are embedded inside a 3D biomimetic ECM network (black lines). Cells in close proximity are able to polarize, migrate, proliferate or differentiate in response to the cytokine gradient. If cells reside far away from the μ-beads and thereby outside the gradient, they will not response (round shape). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Material & methods

2.1. Chemicals

Cell culture grade phosphate buffered saline (PBS) was purchased from Biochrom (Berlin, Germany). Bovine serum albumin (BSA, >98%, M_W = 66 kDa, heat shock fraction), heparin (sodium salt from the porcine intestinal mucosa), lysozyme from chicken hen egg white (>90%, M_W = 14.3 kDa), monosodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$, BioXtra), sodium phosphate dibasic (Na_2HPO_4, BioXtra), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), and N-hydroxysuccinimide (NHS) were all purchased from Sigma-Aldrich (Steinheim, Germany). Sodium bicarbonate ($NaHCO_3$) was purchased from VWR (Darmstadt, Germany), sodium azide (NaN_3, pure) and sodium chloride (NaCl, pure) from AppliChem (Darmstadt, Germany), EDC for μ-bead modification from Merck Chemicals (Darmstadt, Germany), rat tail collagen I (4.1 mg/ml, 3298599) from Corning (Amsterdam, Netherlands), and heparin-FITC from Invitrogen (Darmstadt, Germany). All chemicals were used without further purification. Solutions were prepared with deionized water (ρ = 18.2 MΩ).

For μ-bead modification different GAG were used, see also summary in Table 1. Native high molecular weight HA (from *Streptococcus*, average molecular weight as determined with laser light scattering $M_w = 1.1 \cdot 10^6$ g/mol, polydispersity index PD = 4.8) was obtained from Aqua Biochem (Dessau, Germany), sulfur trioxide/dimethylformamide complex (SO_3-DMF, purum, ≥97%, active SO_3 ≥ 48%) from Fluka Chemie (Buchs, Switzerland). Fluorescence marker (Atto-565-NH_2) was purchased from ATTO-TEC (Siegen, Germany).

The high-sulfated (hsHA) and the medium-sulfated (msHA) HA derivatives were synthesized and characterized as described previously [37]. Low molecular weight HA was prepared by ozonolysis of high molecular weight native HA. A 1% aqueous solution of high molecular weight HA was treated with ozone, prepared with an ozone generator COM-AD-02 (ANSEROS Klaus Nonnenmacher, Tübingen, Germany) for 2 h. The ozone concentration amounted to approx. 30 g/m^3 and a flow rate of 20 to 30 l/h was used. Finally, N_2 was passed through the solution for 30 min to expel free ozone. The remaining clear solution was dialyzed against distilled water, lyophilized and dried under vacuum. The HA was obtained with 75 to 85% yield. Analytical data of the HA derivatives (HA, hsHA, msHA) are summarized in Table 1.

The functionalization of the HA derivatives (HA, hsHA) with fluorescence dye (Atto-565-NH_2) was carried out at the reducing end-group of the macromolecules as previously described [38,39]: Briefly, 0.5 mmol of HA and 0.25 mmol of hsHA, respectively, were dissolved in 30 ml of