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Engineering of thermoresponsive gels as a fake metastatic niche

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ABSTRACT

Chemoattraction through the CXCR4-CXCL12 axis has been shown to be an important mechanism to direct circulating tumor cells toward distant sites. The objective of this work was to prepare a fake metastatic niche made up of a gel loaded with CXCL12. The gel is designed to create a steep concentration gradient of the chemokine in the proximity of the site of administration/injection, aimed to divert and capture circulating $CXCR4^+$ tumor cells. To this aim, different thermoresponsive gels based on methylcellulose (MC) or poloxamers, loaded with CXCL12, with or without hyaluronic acid (HA) were designed and their mechanical properties correlated with the ability to attract and capture *in vitro* $CXCR4^+$ cells. Results of *in vitro* cell studies showed that all prepared gels induced CEM tumor cell migration whereas only gels based on MC embedded with CXCL12 are able to capture them.

1. Introduction

Metastasis can be defined as the dissemination of cancer cells away from the site of origin. The pivotal stages of this process can be schematized as in the following: (i) loss of cell–cell contact in the tumor site; (ii) degradation of the basal lamina by malignant cells, resulting in local invasion; (iii) intravasation of cancer cells into the bloodstream and/or lymphatic system; (iv) migration of cancer cells to other organs and tissues; (v) creation of a pre-metastatic niche where metastasis may form if a permissive microenvironment for tumor cell colonization and proliferation is present (Arvelo, Sojo, & Cotte, 2016; De la Fuente et al., 2015). Cell chemotaxis and subsequent migration toward distal sites is mediated by chemokines, which are a family of small proteins (70–100 amino acids residues) classified according to the number and spacing of their N-terminal cysteines (C). In CXC chemokines, the C residues are separated by another amino acid (X). Chemoattraction of circulating tumor cells through the receptor/ligand axis between CXC chemokine receptor type 4 (CXCR4) and the CXC motif chemokine ligand 12 (CXCL12) has been shown to be an important mechanism to direct circulating tumor cells toward the host organ (Liu, Long, Huang, Sun, & Wang, 2016), and may therefore create opportunities for breakthroughs in cancer therapy. More specifically, CXCL12, also known as stromal cell-derived factor 1 (SDF-1), is a highly effective chemokine with a marked action as a chemotactic factor for T-lymphocytes and monocytes. Furthermore, SDF-1 induces intracellular actin polymerization in lymphocytes, a process that is thought to be a prerequisite for cell motility (Bleul, Fuhlbrigge, Casasnovas, Aiuti, & Springer, 1996). Also CXCL12 plays a major role in tumor metastasis: indeed, cancer cells expressing CXCR4 receptor are attracted toward tissues releasing CXCL12, which therefore becomes a target for metastasis formation (Müller et al., 2001). This phenomenon is crucial to metastasis development since the expression of CXCR4 receptor is present in a wide array of cancer types, including breast, ovarian, melanoma and prostate cancer (Sun et al., 2010).

In this work we have investigated the use of a fake metastatic niche consisting of a gel loaded with CXCL12 and designed to create a steep concentration gradient of the chemokine in the proximity of the site of administration/injection that can divert and recruit $CXCR4^+$ cells. Blocking CXCL12/CXCR4 interactions inhibits downstream intracellular signaling cascades that lead to metastasis development. Specifically, we hypothesize that both the chemical composition and mechanical properties of such gels can affect their ability to create *in situ* a chemical gradient of the chemokine and, thus, their ability to function as a "metastasis trap". Consequently, we have designed different thermoresponsive gels based on methylcellulose (MC) or poloxamers, with or without the addition of hyaluronic acid (HA). The gels were loaded with CXCL12 and their mechanical properties correlated with the *in vitro* ability to attract and capture $CXCR4^+$ cells (CEM).

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Thermoresponsiveness is a pivotal attribute in this context; indeed, thermoresponsive polymers exhibit a drastic change in their mechanical/physical properties after small changes of temperature around a critical value known as lower critical solution transition temperature (LCST). In particular, the gels were designed to have a LCST close to the physiologic temperature. Thus, the matrices can be administered by means of a syringe being a viscous solution at 25 °C but, once at body temperature, they are able to form *in situ* a gel with mechanical properties suitable to capture $CXCR4^+$ circulating tumor cells (Klouda & Mikos, 2008). Cellulose derivatives are examples of thermosensitive polymers and, in particular, aqueous solutions of MC, at low concentrations (1–10 wt%), are known to be liquid at low temperature but able to form a gel upon heating up to 40–50 °C. MC gelation temperature can be engineered so as to be close to the physiological temperature, by blending it with other polymers such as HA (Mayol et al., 2014). HA is a natural mucoadhesive polysaccharide, widely used in drug delivery and biomedical field, composed of alternating D-glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine (GlcNAc) repeating units linked together *via* β-(1,4) and β-(1,3) glycosidic junctions. Polymeric blends represent an important class of biomaterials since they can combine the advantages of the polymers utilized. In particular, in the case of MC/HA blends, the thermosensitive properties of MC can be implemented with the well-known biocompatibility and mucoadhesivity features of HA. Similarly, another class of thermoresponsive gels widely explored in controlled drug delivery are poloxamers (POLOX), which are a class of amphiphilic triblock copolymers made up of hydrophilic polyethylene oxide (PEO) and hydrophobic polypropylene oxide (PPO) along their backbone, with a PEO-PPO-PEO architecture. Nevertheless, these gels possess inadequate mechanical properties, poor bioadhesiveness and high permeability to water. To overcome these issues, we blended HA with poloxamer analogs (Mayol et al., 2011).

2. Materials and methods

2.1. Materials

MC with an approximate viscosity equal to 4000 cP at 2% (w/w) and at 20 °C, Mn and Mw equal to 45 kDa and 196 kDa, respectively and a 1.5–1.9 ° of substitution (data provided by the supplier) was purchased from Sigma (Milano, Italy). POLOX with variable numbers of PEO (a) PPO (b) units were used. POLOX F127 (a = 100 and b = 65), with a Mw of 12.6 kDa and F68 (a = 76 and b = 29), with a Mw of 8.4 kDa, were obtained from Lutrol (Basf, Germany). HA with Mn and Mw equal to 482 kDa and 833 kDa respectively, was produced by Novozymes Biopharma DK A/S by fermentation of the strain *Bacillus subtilis* (Borzacchiello, Russo, Malle, Schwach-Abdellaoui, & Ambrosio, 2015). MC and HA molecular weight were measured by Size Exclusion Chromatography (SEC) using a HP1100 Chemstation (Agilent, USA) equipped with refractive index signal as detector. More in detail, we used a combination of two columns in series: Superchrome Biobasic SEC300 (300 × 7.8 mm, 5 µm, 300 Å) and 120 (300 × 7.8 mm, 5 µm, 120 Å), at the flow rate of 0.4 mL/min and 35 °C. Samples at the concentration of 5 mg/mL were eluted using a mobile phase constituted of pH 6.8 0.05 M PB and 0.25 M KCl. For each sample the injection volume was 50 µL. The Mw of each sample was calculated using dextrans at different known molecular weights as calibration standards. Potassium chloride (KCl) from Carlo Erba (Milano, Italy), dibasic sodium phosphate (Na_2HPO_4), sodium chloride (NaCl) from Sigma-Aldrich (St. Louis, USA) were used. CXCL12 was purchased from R&D systems (Minneapolis, USA).

2.2. Preparation of MC and MC-HA gels

A Gel made only of MC was prepared by dissolving MC (2% w/v) in phosphate buffered saline (PBS, 120 mM NaCl, 2.7 mM KCl, 10 mM phosphate salts, pH = 7.4), as previously reported (Mayol et al., 2014). Briefly, the solvent was divided into two aliquots of equal volume: one was cooled to 0 °C and the other one was heated to the boiling point. MC was slowly solubilized in the hot solvent and then the cold solvent was added under magnetic stirring for about 4 h, in an ice bath. The resultant solution was kept at 4 °C overnight. MC-HA gel was obtained by simply adding HA (0.1% w/v) into MC gel and mixing the resultant solution for one hour. For *in vitro* migration assay, CXCL12 was merely dispersed into the gel at a concentration that has proved to be effective in previous work (0.00003% w/v) (Glodek et al., 2007).

2.3. Preparation of POLOX and POLOX-HA based gel

POLOX gel was prepared as previously described with some modifications (Mayol et al., 2011). Briefly, POLOX F127 and F68 (21.43% w/v each) were mixed in distilled water under magnetic stirring, in an ice bath, until a clear solution was obtained. For complete solubilization, the solution was kept at 4 °C overnight. POLOX-HA based formulation was obtained by adding HA (0.1% w/v) within the POLOX solution under continuous stirring. The gels were stored at 4 °C until use. For *in vitro* migration assay, CXCL12 was merely dispersed into the gel.

2.4. Cell culture

CCRF-CEM, human T-Leukemia cells, named CEM cells in the following, were grown in Roswell Park Memorial Institute (RPMI) – 1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, Utah, USA) with 10% fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, penicillin and streptomycin (50 µg/mL each).

2.5. Cell migration assay

Cell migration was assayed in 24-well Transwell® chambers (Corning Inc., Corning, NY) using inserts with 6.5-µm pore membrane. CEM cells, pre-stained with live dye green cell tracker (CellTracker Green CMFDA, Invitrogen, USA) were placed in the upper chamber (2.0×10^5cells/well) in the presence of a migration medium made of a 0.5% w/v BSA solution in 500 µL RPMI. The lower chamber contained also the gels with or without CXCL12, bare cell culture medium as a control or a CXCL12 solution in RPMI at 300. After 4 h of incubation, cells on the upper surface of the filter were removed using a cotton wool swab; the membrane was then fixed in 4% paraformaldehyde for 15 min and the cells migrated toward the lower surface of the membrane were stained with DAPI (4,6-Diamidino-2phenylindole, 1:25000, AbD Serotec, UK), photographed and visually counted in 10 random selection of fluorescent microscope fields (Carlo Zeiss, Axio Scope.A1). Triplicate experiments were carried out. The amount of migrated cells was estimated through the migration index, defined as the ratio between number of migrating cells toward BSA containing media (CXCL12-loaded gels, unloaded gels or CXCL12/BSA).

2.6. Rheological experiments

The rheological properties of the gels were evaluated on a rotational rheometer (Malvern Kinexus) using a cone and plate geometry. In particular, the viscoelastic response of each formulation was evaluated by small-amplitude oscillatory shear tests. Briefly, each experiment was performed at 25 °C and 37 °C, in the 0.1–10 Hz oscillation frequency range with a shear strain fixed at which linear viscoelasticity is attained. The solutions were placed between the two plates with a gap < 1 mm. Thus, the shear storage or elastic modulus (G') and the shear loss or viscous modulus (G") were measured as a function of frequency, which respectively characterize the solid-like contributions or the energy stored in the material during deformation and fluid-like contributions, or energy dissipated as heat, to the measured stress response.