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Chitosan thermogels for local expansion and delivery of tumor-specific T lymphocytes towards enhanced cancer immunotherapies

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ABSTRACT

The success of promising anti-cancer adoptive cell therapies relies on the abilities of the perfused $CD8^+$ T lymphocytes to gain access to and persist within the tumor microenvironment to carry out their cytotoxic functions. We propose a new method for their local delivery as a living concentrate, which may not only reduce the numbers of cells required for treatment but also enhance their site-specific mobilization. Using combinations of sodium hydrogen carbonate and phosphate buffer as gelling agents, novel injectable chitosan-based biocompatible thermogels (CTGels) having excellent mechanical properties and cytocompatibility have been developed. Three thermogel formulations with acceptable physico-chemical properties, such as physiological pH and osmolality, macroporosity, and gelation rates were compared. The CTGel2 formulation outperformed the others by providing an environment suitable for the encapsulation of viable $CD8^+$ T lymphocytes, supporting their proliferation and gradual release. In addition, the encapsulated T cell phenotypes were influenced by surrounding conditions and by tumor cells, while maintaining their capacity to kill tumor cells. This strongly suggests that cells encapsulated in this formulation retain their anti-cancer functions, and that this locally injectable hydrogel may be further developed to complement a wide variety of existing immunotherapies.

1. Introduction

Systemic adoptive cell therapy (ACT) is an emerging form of cancer immunotherapy showing tremendous potential in clinical studies aimed at treating solid cancers. The success of ACT relies on the expansion of tumor infiltrating lymphocytes (TIL) from patient-derived tumors for their personalized systemic infusion back into individual patients, along with the capacity of these antigen-experienced and activated TIL to access and persist in the tumor microenvironment where they may elicit their cytotoxic, anti-cancer responses [1]. Three main pitfalls of the current clinical protocol keep this promising form of treatment from becoming mainstream: 1) ACT requires the expansion of vast numbers of patient-tumor derived TIL (20–150 billion) for the final GMP-grade infusion product [2], and which, when not possible causes a great number of patients to lose treatment eligibility [3]. 2) TIL expansion using a rapid expansion protocol (REP) over many weeks causes a growth-mediated outcompeting/diminishment in the proportion tumor-reactive cells [4,5], and the extended period of time associated to "selected TILs" protocol often does not match with the rapid deterioration of patients [6,7]. Though the "young TIL" expansion protocols produce more reactive TIL [4,5,8–10], this measure also limits patient eligibility when expanded TIL do not reach the therapeutic numbers required for ACT. 3) Large numbers of TIL must be expanded because their systemic delivery causes many to be lost to non-cancerous sites of inflammation such as the pulmonary micro-vasculature [11,12]. Another drawback of this anti-cancer treatment is the toxic effects produced by the high-dose bolus interleukin-2 (IL-2) administrated to maintain the TIL in an activated state following ACT [13]. It is clear that the pitfalls of the current TIL-ACT protocol are the result of the systemic TIL administration protocol. This has led us to develop a new method for the

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local injection of concentrated, three dimensional (3D) TIL cultures into the tumor microenvironment akin to adjuvant postoperative radiation therapy currently applied to cavities formed from tumor resection in cases of advanced tumor stage and margin positivity, and also with the intent to mimic naturally occurring tertiary lymphoid structures associated to positive patient prognosis [14–20].

We hypothesize that the implantation of 3D T cell cultures will provide a means for the delivery of a continuous feed of these cells towards the reduction of tumor burden or of tumor reoccurrence near the site of tumor resection. To that end, the molecular architecture of the scaffold must allow the proliferation and release of functional T cells whose activation state can be influenced by the surrounding conditions, allowing activating and chemoattractant signatures from the tumor microenvironment to accelerate the proliferation, escape, and immunogenicity of the encapsulated cells. It must also allow its precise, local administration in a minimally invasive way (through catheter or needle), its gellifying ahead of its dispersion, and must provide it with the ability to withstand *in vivo* stress and degradation for the duration of treatment.

Towards this purpose, chitosan thermogels were developed to act as injectable matrices supporting cellular growth. Chitosan, a deacetylated derivative of chitin, is a natural, biocompatible, non-immunogenic and biodegradable polysaccharide which has been examined for use towards a wide range of biomedical applications [21–23]. When combined with a weak base such as β-glycerophosphate (BGP), it can form a solution (a thermogel) having physiological pH and which gelates at 37 °C [24]. Though this thermogel has been considered for several biomedical applications, its mechanical properties are very poor and its biocompatibility is limited. Indeed, we and others have shown that the concentration of BGP required to reach rapid gelation (0.4 M) is cytotoxic to cells due to its hyperosmolality [25,26]. Culture medium incubated with CH-BGP hydrogels prepared with BGP of 0.4 M or above had a cytotoxic effect on cells [26]. Moreover, direct encapsulation of L929 mouse fibroblasts led to poor cell viability and growth (data not shown). These limitations were recently overcome by replacing BGP with a combination of sodium hydrogen carbonate ($NaHCO_3$, hereafter called SHC) and phosphate buffer (PB) [26]. This combination greatly enhanced the mechanical properties of the chitosan hydrogel, while keeping its thermosensitive properties, rapid gelation at 37 °C and low salt concentration, suggesting that it might be ideal for cell encapsulation. In addition to its key role in enhancement of gel mechanical properties, SHC is a porogen which concentration has been shown to influence hydrogel porosity [27]. Indeed, when mixed with an acidic chitosan solution the reaction between SHC (HCO_3^-) and protons (H^+) leads to the generation of carbon dioxide (CO_2) [27,28]. Thus our previous work showed that the final concentration of both gelling agents influences gelation kinetic and porosity, and the modulation of their ratios allows the thermogel formulation to be adjusted according to the specific needs of individual clinical applications [26].

In this study, three different formulations have been investigated with the aim to create injectable chitosan thermogels of different morphologies capable of housing the growth of 3D cultures of activated T lymphocytes. Based on our previous results [26], the concentration of PB was fixed to 0.04 M to reach adequate gelation kinetics and injectability, and three different concentrations of SHC were used to obtain hydrogels with different porosities influencing cell proliferation and release: SHC 0.05 M (CTGel1), SHC 0.075 M (CTGel2) and SHC 0.12 M (CTGel3).

CTGel rheological properties and mechanical strengths were evaluated by rheometry and unconfined compression tests, respectively, and their morphologies were compared by scanning electron microscopy (SEM). CTGel cytocompatibility was determined from the encapsulation of primary T cells expanded from normal donor peripheral blood mononuclear cells (PBMCs). CTGel and supernatant-derived cells numbers, cell viability, phenotype and activation status were recorded over time using microscopy and flow cytometry. The CTGel2 formulation was found to be superior to the others and was further studied to reveal its potential for cancer immunotherapy, as demonstrated from the activation and escape of encapsulated TIL and T cells in response to, and for the killing of their cognate tumor cells.

2. Experimental methods

2.1. Hydrogels preparation

Chitosan (Marinard Biotech, Mw 250 kDa, DDA 94%) was purified using sodium dodecyl sulfate as previously described [26]. Chitosan solution was then obtained by solubilizing purified chitosan in HCl (0.1 M) at 3.33% (w/v) overnight at room temperature. The resulting chitosan solution was sterilized by autoclaving (20 min, 121 °C) and was stored at 4 °C until further use. Gelling agent solutions were prepared by mixing SHC and PB at pH 8 (prepared by mixing sodium phosphate dibasic (Na_2HPO_4, SPD) and sodium phosphate monobasic (NaH_2PO_4, SPM) at a ratio of 0.932:0.068) in Milli-Q water (EMD Millipore). These were prepared to obtain hydrogels with a final PB:SHC concentration of 0.04 M:0.05 M (CTGel1), 0.04 M:0.075 M (CTGel2) and 0.04 M:0.12 M (CTGel3). Gelling agents were sterilized by filtration through 0.2 μm filters and were stored at 4 °C until further use.

All CTGels were prepared to reach a final chitosan concentration of 2% (v/w). Chitosan solution (3.33%, v/w) was loaded into a syringe and the gelling agent solution was loaded into another (volume ratio of 3:2). For mixing, the two syringes were joined by a luer lock connector (Qosina, USA), and syringe contents were pushed from side to side for 15 repeats.

2.2. Morphology, pH and osmolality of injectable hydrogels

After 24 h of incubation at 37 °C, hydrogels were squeezed through a 0.2 μm filter. The pH of the recovered liquid was then determined using a pH-meter (UltraBasic pH-meter, Denver Instrument, USA) and the osmolality was determined using an AdvancedTM Micro Osmometer 3300 (Advanced Instruments Inc., Norwood, USA). For SEM analysis, gels were frozen at −20 °C, freeze-dried, cut into sections using a surgical scalpel, and deposited onto double-coated carbon conductive tape before being metalized with gold. Their morphologies were then analyzed using a Hitachi S-3600 SEM.

2.3. Rheological properties and mechanical strength

The gelation kinetics of CTGels at physiological (37 °C) and room (22 °C) temperature were studied by following their rheological properties using a Physica MCR 301 (Anton Paar, Germany) equipped with coaxial cylinder geometry (CC10/T200). The evolution of the storage (G′) and loss (G″) moduli was determined in the linear viscoelastic range at a constant shear stress (1 Pa) and at a constant frequency (1 Hz) over the course of 60 min. The time at which $G' = G''$ represents the gelation time [29] and changes in G′ indicate the progressive gelation and increase of the elastic properties of the gel over time. The Young's modulus and mechanical strength in compression after 24 h of gelation at 37 °C were determined using an ElectroForce 3200 test instrument (Bose Corporation, USA) with a 22 N load cell. Samples were prepared in 14 mm inner diameter cylinder molds. Unconfined axial