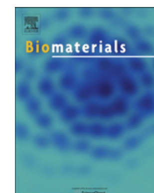
Biomaterials 30 (2009) 6757–6767

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials



Engulfing tumors with synthetic extracellular matrices for cancer immunotherapy

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ARTICLE INFO

Article history:
Received 15 June 2009
Accepted 26 August 2009
Available online 19 September 2009

Keywords:
Local tumor immunotherapy
IL-15 superagonist
Dendritic cells
Alginate
Hydrogels
Cancer vaccines

ABSTRACT

Local immunotherapies are under investigation for the treatment of unresectable tumors and sites of solid tumor resection to prevent local recurrence. Successful local therapy could also theoretically elicit systemic immune responses against cancer. Here we explored the delivery of therapeutic dendritic cells (DCs), cytokines, or other immunostimulatory factors to tumors via the use of 'self-gelling' hydrogels based on the polysaccharide alginate, injected peritumorally around established melanoma lesions. Peritumoral injection of alginate matrices loaded with DCs and/or an interleukin-15 superagonist (IL-15SA) around 14-day established ova-expressing B16F0 murine melanoma tumors promoted immune cell accumulation in the peritumoral matrix, and matrix infiltration correlated with tumor infiltration by leukocytes. Single injections of IL-15SA-carrying gels concentrated the cytokine in the tumor site ~40-fold compared to systemic injection and enabled a majority of treated animals to suppress tumor growth for a week or more. Further, we found that single injections of alginate matrices loaded with IL-15SA and the Toll-like receptor ligand CpG or two injections of gels carrying IL-15SA alone could elicit comparable anti-tumor activity without the need for exogenous DCs. Thus, injectable alginate gels offer an attractive platform for local tumor immunotherapy, and facilitate combinatorial treatments designed to promote immune responses locally at a tumor site while limiting systemic exposure to potent immunomodulatory factors.

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1. Introduction

Localized cancer therapies are utilized to treat unresectable tumors and/or for treating sites of surgical resection to combat local recurrence [1–3]. Immunotherapy treatments delivered directly at solid tumor sites have been explored with the goal of utilizing the tumor itself as a source of tumor antigens to generate a systemic immune response that could eliminate distal metastases. Most immunotherapy strategies seek to promote the robust infiltration of tumors with functional immune cells to promote tumor destruction, but defects in tumor vasculature, suppressive signals produced by tumor cells or co-opted tumor-resident immune cells, and rapid tumor growth can limit the accumulation of activated and competent immune cells [4,5]. Recently, combinations of cytokine, chemotherapy, and/or immunostimulatory ligand treatments used to locally treat established tumors have shown promise in not only eliminating treated tumors but also generating systemic immunity [6–11]. However, such potent immunostimulatory regimens can elicit serious toxicity and may need to be coupled with methods to control delivery and limit systemic exposure [12,13]. Prior work has demonstrated that controlled release of immunocytokines from gels or microparticles at a tumor site can enhance local immunotherapies, by sustaining the intratumoral concentration of these factors while reducing systemic exposure following a single injection [14–16]. Thus, the use of biomaterials to deliver local combinatorial immunotherapies may lead to further enhancements in the potency and safety of local immunotherapy.

We recently described an injectable gel formulation of the polysaccharide alginate, which can be loaded with exogenous immune cells, proteins, or immunoregulatory factors [17,18]. Alginate has been studied extensively as a matrix for cell therapy and tissue engineering, and has been shown to be safe for use in humans [19–21]. We found that injection of alginate gels with embedded activated dendritic cells in healthy mice elicits a sustained infiltration of host T-cells and dendritic cells into the matrix, and that these matrices can release encapsulated cytokines over a period of 7–14 days [17,18]. We hypothesized that a similar recruitment of lymphocytes and DCs to alginate gels surrounding

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doi:10.1016/j.biomaterials.2009.08.037

established tumors could promote local antigen presentation, and provide a local reservoir of immune cells for tumor invasion. To test this concept, in the present study we surrounded established melanoma tumors with DC/cytokine/Toll-like receptor (TLR) ligand-loaded gels, and analyzed tumor growth and the recruitment of leukocytes to the tumor-engulfing gels and the tumors themselves. Because these gels were stable at least one month *in vivo* they were readily recovered post-treatment, and thus we also analyzed the composition of innate and adaptive immune cells recruited to both the gels and the tumor itself following peritumoral gel therapy.

2. Materials and methods

2.1. Materials

Sterile alginates Pronova SLM20 (MW 75,000–220,000 g/mol, >50% M units) and Pronova SLG20 (MW 75,000–220,000 g/mol, >60% G units) were purchased from Novamatrix (FMC Biopolymers, Sandvika, Norway). Anti-mouse FITC-TCRβ, anti-mouse PE-I-A^b, anti-mouse APC-CD11c, anti-mouse APC-CD8α, anti-mouse APC-CD4, anti-moue FITC- and PE-NK1.1 were purchased from BD Biosciences (San Jose, CA). APC-tetramer ova-MHC I was from Beckman Coulter (Fullerton, CA), anti-mouse PE-CD8α antibody was from Invitrogen (San Diego, CA), and anti-mouse foxp3 staining kit was from eBioscience (San Diego, CA). Isooctane was obtained from Mallinckrodt Baker (Phillipsburg, NJ). Calcium chloride dihydrate and alginate lyase were from Sigma–Aldrich (St. Louis, MO). CpG oligonucleotides with a phosphorothioate backbone (CpG 1826, sequence 5′-/5AmMC6/TCCATGACGTTCCT-GACGTT-3′) were synthesized by Integrated DNA Technologies. Mouse IL-15 and mouse IL-15Rα-human Fc chimera recombinant proteins and ELISA detection kits were purchased from R&D Systems (Minneapolis, MN).

2.2. Animals and cells

Animals were cared for in the USDA-inspected MIT Animal Facility under federal, state, local and NIH guidelines for animal care. C57Bl/6 mice were obtained from the Jackson Laboratory. Bone marrow-derived dendritic cells were prepared following a modification of the procedure of Inaba [22] as previously reported [23]. DCs were activated/matured with 1 μM CpG and pulsed with 1 μg/mL each of ova class I and ova class II peptides (Anaspec, San Jose, CA) for 18 h and washed 3X with PBS before use. B16F10 parental melanoma cells were obtained from American Type Culture Collection. B16-ova cells based on the B16F0 parental melanoma line were transfected with ovalbumin expressed as an MSCV vector with puromycin resistance, followed by an IRES and Ovalbumin-2A-green fluorescent protein (GFP). The ova expressed in these cells lacks the first 55 amino acids (deleting the secretion signal) and are soluble in the cytoplasm.

2.3. Tumor inoculation and alginate gel therapy

Except where noted otherwise, anesthetized C57Bl/6 mice were inoculated with 3×10^4 B16-ova cells s.c., which were allowed to establish for 14 days. IL-15 superagonist (IL-15SA) was prepared by incubating 5 μg IL-15 and 31.7 μg IL-15Ra/Fc (equimolar amounts) at 37 °C in 15.9 μL PBS for 30 min. Matrix alginate (160 μL of 0.01 g/mL SLM20 alginate in sterile PBS at 4 °C) was mixed with factors to be delivered (e.g., 2×10^6 dendritic cells, 36.7 μg IL-15 SA, and/or 80 μg CpG in 150 μL gel) and kept on ice until injection. Calcium-loaded alginate microspheres were synthesized as previously described [17,18]. Endotoxin levels in the alginate preparations were well below levels stimulatory for innate immune cells [24], as described previously [18]. The Calcium-loaded microspheres ($\sim 1 \times 10^6$) were mixed with the matrix alginate solution and 150 μL of the mixture immediately injected s.c., surrounding tumors. For i.p. injections, 36.7 μg of IL-15SA was injected in 200 μL of PBS, and for i.t. injections, the same amount of IL-15SA and 2×10^6 dendritic cells were injected into tumors in 30 μL of PBS.

2.4. Flow cytometry analysis

Alginate gels, lymph nodes, and spleens recovered from treated animals were digested with 0.28 WÜ/mL Liberase Blendzyme 3 (Roche Applied Sciences, Indianapolis, IN) and 1 mg/mL of alginate lyase (Sigma) for 20 min at 37 °C. Digested gels and tissues were passed through a 40 μm nylon mesh cell strainer (BD Falcon) with 9 mL complete RPMI medium. Recovered cells were resuspended in FACS buffer (1% BSA, 0.1% NaN_3 in Hank's balanced salt solution, pH 7.4) at 4 °C, blocked with anti-CD16/32 antibody for 10 min, then stained with fluorescent antibodies for 20 min on ice, followed by 3 washes with FACS buffer and addition of 1.25 μg/mL propidium iodide (PI) for viability assessment. For foxp3 staining, cells were fixed, permealized, and stained according to the manufacturer's instructions. Stained cells were analyzed on a BD FACSCalibur flow cytometer. Enumeration of cellular infiltrates was performed by calibration of flow cytometer events to cell suspensions of known concentration, and cell frequencies determined from a live cell gate with low PI staining. Cell losses during the multiple wash/treatment steps of gel and tissue digestions were reproducible and were accounted for in the reported recovered cell numbers.

2.5. IL-15SA release in vitro and in vivo

Soluble IL-15Rα/Fc (15.9 μg, 2 mg/mL in PBS) was mixed with 80 μL of alginate matrix solution (0.01 g/mL in PBS), and then gelled with 5×10^5 calcium-loaded alginate microspheres to cross-link the alginate in an Eppendorf tube for 2 h at 37 °C. RPMI medium with 10% FCS was added (500 μL) and cytokine release was assessed by ELISA analysis quantifying IL-15Rα in the supernatant at staggered time points. The release of the IL-15SA was quantified *in vivo* by injecting alginate gels carrying 36.7 μg IL-15SA (31.7 μg IL-15Rα-Fc + 5 μg IL-15) around 14 day-old B16-ova tumors in C57Bl/6 mice. Gels and tumors were recovered from independent mice at staggered times, digested using the protocol described above, or by using T-Per Tissue Protein Extraction Reagent (Pierce, Rockford, IL) supplemented by Halt Protease Inhibitor Cocktail (Pierce) according to the manufacturer's instructions. The digestion supernatants were collected and the amount of IL-15SA was quantified by using IL-15Rα ELISA kit or two-site IL-15Rα/IL-15 ELISA. IL-15 loss due to the digestion process was analyzed using control digestions using known quantities of IL-15SA and were accounted for in the analysis.

2.6. Statistical analysis

All data are shown as mean ± S.E. Comparisons of two experimental groups were performed using two-tailed Mann–Whitney tests. Comparisons of Kaplan–Meier survival curves were made using a log-rank test.

3. Results

3.1. T-cell recruitment to tumor-surrounding gels

We recently developed an injectable 'self-gelling' formulation of the polysaccharide alginate, obtained by mixing calcium-loaded alginate microspheres with an alginate solution just prior to injection [17,18]. Upon injection, calcium ions from the microspheres and the surrounding interstitial fluid diffuse into the alginate solution and ionically cross-link the polysaccharide chains *in situ*, enabling gelation in <60 min *in vivo*. The resulting soft gels retain more than 80% of their original mass for ∼30 days *in vivo*, and can entrap co-injected cells or cytokines for slow release into the local environment [17,18]. In our previous study in healthy animals [17], we showed that s.c. injection of alginate gels carrying activated, antigen-loaded dendritic cells (DCs) elicited priming of naïve CD8$^+$ T-cells in the local draining lymph nodes (supported by migration of a small number of DCs from the gel to the lymph nodes), followed by trafficking of primed antigen-specific T-cells to the gels. To determine whether DCs delivered in alginate gels could similarly promote accumulation of antigen-specific T-cells at solid tumor sites, we analyzed the response of established melanoma tumors to locally-injected DC-carrying gels. We generated a model system where B16F0 melanoma cells were transfected with green fluorescent protein (GFP) and ovalbumin to enable visualization of antigen capture by dendritic cells and tracking of the immune response to a defined antigen, respectively. We first analyzed the response of established GFP/ova-expressing B16F0 (hereafter designated B16-ova) tumors to peritumoral injections of DCs in alginate gels. B16-ova cells were inoculated s.c. in C57Bl/6 recipients and allowed to establish for 7 or 14 days. Mice then received a single injection of alginate (150 μL) to surround tumors with gels carrying activated bone marrow-derived dendritic cells loaded with immunodominant class I- and class II-restricted ova peptides (Fig. 1A). Tumor growth was monitored and gels were recovered 14 days after injection for digestion and analysis of the local immune cell infiltrate accumulating at the tumor site. As shown in Fig. 1B, "empty" alginate gels injected around 14-day-old tumors contained few CD4$^+$ or CD8$^+$ T-cells. In contrast, gels carrying ova-pulsed, activated DCs elicited a substantial accumulation of CD4$^+$

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