Biomaterials 34 (2013) 3828–3836

Contents lists available at SciVerse ScienceDirect

Biomaterials

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

Role of chitosan co-formulation in enhancing interleukin-12 delivery and antitumor activity

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ARTICLE INFO

Article history:
Received 30 January 2013
Accepted 10 February 2013
Available online 27 February 2013

Keywords:
Chitosan
Paracrine delivery
Interleukin-12
Intratumoral
Immunotherapy

ABSTRACT

Local delivery systems that provide sustained, high concentrations of antitumor cytokines in the tumor microenvironment while minimizing systemic dissemination are needed to realize the potential of cytokine-based immunotherapies. Recently, co-formulations of cytokines with chitosan solutions have been shown to increase local cytokine retention and bioactivity. In particular, intratumoral (i.t.) injections of chitosan/IL-12 can eliminate established tumors and generate tumor-specific immune responses. In the present study, we explored the mechanisms by which chitosan potentiated IL-12's antitumor activity. The location of chitosan/IL-12 injection was found to be critical for optimal cytokine delivery. I.t. injections eliminated 9 of 10 MC38 adenocarcinomas while contralateral and peritumoral injections delayed tumor growth but could not eliminate tumors. Microdosing studies demonstrated that IL-12 depots, simulated through daily i.t. injections with IL-12 alone, were not as effective as weekly i.t. chitosan/IL-12. 50–75% of mice receiving daily IL-12 microdoses and 87.5% of mice receiving weekly chitosan/IL-12 were cured of MC38 tumors. Chitosan was found to increase IL-12-mediated leukocytic expansion in tumors and tumor-draining lymph nodes (TDLNs) by 40 and 100%, respectively. Immunophenotyping studies demonstrated that chitosan co-formulation amplified IL-12-induced increases in important effector populations, such as $CD8^{+}IFN\text{-}\gamma^{+}$ and NKT cells, in tumors and dendritic cell populations in TDLNs. Remarkable increases in $Gr\text{-}1^{+}CD11b^{+}$ tumor infiltrates were also observed in mice receiving chitosan or chitosan/IL-12. This population does not appear be suppressive and may facilitate the local antitumor response. Presented data suggest that chitosan-mediated depot formation and enhanced local cytokine retention is significantly, but not entirely, responsible for increased cytokine bioactivity.

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1. Introduction

Since the discovery of 'endogenous pyrogen,' now known as IL-1, in 1953 [1], the use of exogenous cytokines to treat malignant neoplasms has been well studied and heavily pursued. However, only 2 of 40+ identified cytokines, IFN-α and IL-2, are approved as single agent immunotherapies for a limited number of indications. IFN-α therapy yields an 80% overall response rate with 10% complete responses in hairy cell leukemia [2], 40% objective response rate in AIDS-related Kaposi's sarcoma [3,4], and 10–20% complete response rate in chronic myelogenous leukemia [5,6]. IL-2 therapy yields 10–20% overall response rates with about 5% complete responses in both metastatic renal cell carcinoma [7–9] and metastatic melanoma [8,10,11].

Both IFN-α and IL-2 are administered as systemic injections and cause significant adverse events. In particular, systemic IL-2 often requires intensive care due to grade 3 and 4 adverse events including fever, transaminase elevation, hypotension and edema. Nearly all clinical trials, both past and present, evaluating cytokine monotherapies utilize systemic (i.v., s.c. or i.m.) injections. However, cytokines function primarily through paracrine and autocrine mechanisms and thus are rarely measurable in the circulation of healthy individuals.

We and others have noted that cytokine-based immunotherapies would be more effective and less toxic if delivered locally and maintained in a tissue of interest, i.e. the tumor. Furthermore, a growing mountain of evidence demonstrates that locally administered cytokines can generate adaptive immunological memory capable of controlling metastasis and preventing recurrence [12–14]. This "local-to-systemic" antitumor immunity encourages re-evaluation of systemic cytokine delivery and justifies the development of localized delivery strategies capable of maximizing cytokine delivery to the tumor microenvironment while

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http://dx.doi.org/10.1016/j.biomaterials.2013.02.031

minimizing toxicities associated with systemic dissemination of potent, pro-inflammatory cytokines.

Here, we continue our investigation of simple co-formulations of chitosan solution with recombinant cytokines for local administration. Chitosan is a nontoxic (LD50 > 16 g/kg) [15], biodegradable, natural polysaccharide derived from the exoskeletons of crustaceans. Chitosan is a widely used biomaterial with an established safety profile in humans. It is used as a pharmaceutical excipient [16], a weight-loss supplement, an experimental mucosal adjuvant [17] and in an FDA-approved hemostatic dressing [18].

Our previous studies have demonstrated that simple, viscous chitosan solutions are able to maintain high concentrations of co-formulated recombinant cytokines and/or protein antigens following intratumoral (i.t.) or s.c. administration [19–22]. In particular, i.t. injections of co-formulated chitosan and recombinant IL-12 (chitosan/IL-12) were found to eliminate flank MC38 and Panc02 tumors [22]. The ability of chitosan to enhance the retention of IL-12 in the tumor microenvironment was thought to be primarily responsible for the increased antitumor activity. However, the importance of injecting the depot formulation directly into the tumor was not explored. It is possible that sustained systemic release of IL-12 from a distal site, similar to gene-based IL-12 delivery strategies [23–25], may have produced comparable results. In addition, although chitosan alone exhibited no antitumor activity [21,22], we did not explicitly rule out the possibility that chitosan modulates the immunologic potential of IL-12 through manipulation of local immune cell phenotypes.

The goal of the present study was to understand the mechanisms by which chitosan potentiates cytokine bioactivity. Does chitosan simply amplify cytokine bioactivity through enhanced retention or does chitosan induce an immune response capable of synergizing with cytokine function? To explore the effect of local vs. distal IL-12 depots, we characterized the antitumor activities of chitosan/IL-12 injected adjacent to the tumor, contralateral to the tumor or i.t. To isolate the effect of enhanced IL-12 retention in the tumor on antitumor efficacy, we simulated sustained release of IL-12 from a chitosan-based depot through a series of daily injections with fractionated doses of IL-12 alone. Specifically, weekly i.t. injections of chitosan/IL-12 were replaced by five daily injections with equal or decreasing microdoses of IL-12. Finally, to determine if chitosan potentiates the immunologic activity of IL-12, we performed a phenotypic analysis of immune cell subsets in spleens, tumors and tumor-draining lymph nodes (TDLNs) during immunotherapy.

2. Materials and methods

2.1. Laboratory animals

Female C57BL/6J mice, 8- to 12-weeks old, were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were housed and maintained under pathogen-free conditions in microisolator cages. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Arkansas. Animal care was in compliance with The Guide for Care and Use of Laboratory Animals (National Research Council).

2.2. Reagents

Dulbecco's modified Eagle's Medium (DMEM) was obtained from Hyclone Laboratories (Logan, Utah). Fetal bovine serum (FBS) was purchased from PAA Laboratories (Dartmouth, MA). Trypsin-versene/EDTA, penicillin, streptomycin, nonessential amino acid solution (NEAA), sodium pyruvate, HEPES and ACK lysis buffer were purchased from Lonza (Walkersville, MD). Dulbecco's phosphate-buffered saline (DPBS) and Hanks' balanced salt solution (HBSS) were purchased from Mediatech (Manassas, VA). Histopaque 1077 was obtained from MP Biomedicals (Solon, OH, USA). Cell aggregate dissociation buffer Accumax was purchased from Global Cell Solutions (Charlottesville, VA). Glucose, ethylenediaminetetraacetic acid (EDTA), and bovine serum albumin (BSA) were purchased from Amresco (Solon, OH). Sodium azide was purchased from Alfa Aesar (Ward Hill, MA). FACS buffer was comprised of PBS supplemented with 5 mM EDTA, 0.2% BSA, and 0.2% sodium azide. Chitosan glutamate, 200–600 kDa, 75–90% deacetylated (Protosan G 213) was purchased from NovaMatrix (Sandvika, Norway). Chitosan glutamate was reconstituted in DPBS to a final concentration of 1.5% (*w*/*v*) and henceforth referred to as chitosan solution. Recombinant murine IL-12 was purchased from PeproTech (Rocky Hill, NJ).

Fluorescence-labeled antibodies for flow cytometry were purchased from BD Biosciences (San Diego, CA) and include: anti-CD25 (clone: 7D4), anti-CD54 (clone: 3E2), anti-CD19 (clone: 1D3), anti-FoxP3 (clone: MF23), anti-I-Ab (clone: AF6-120.1), anti-CD3 (clone: 500A2), anti-IFN-γ (clone: XMG1.2), anti-CD11b (clone: M1/70), anti-CD25 (clone: PC61), anti-NK1.1 (clone: PK136), anti-CD45 (clone: 30-F11), anti-CD4 (clone: RM4-5), anti-CD11c (clone: HL3), anti-CD8 (clone: 53-6.7), and anti-Gr-1 (clone: RB6-8C5).

2.3. Cells

The murine colon carcinoma cell line, MC38, was generously provided by Dr. Jeffrey Schlom (Laboratory of Tumor Immunology and Biology, National Cancer Institute, Bethesda, MD). Cells were maintained in DMEM supplemented with 10% FBS, 1% NEAA, 1% HEPES, 1% L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

2.4. Immunotherapy studies

C57BL/6J mice were inoculated s.c. in the shaved flank with 3×10^5 MC38 cells. For antitumor studies investigating the effect of injection location, tumor-bearing mice ($n = 10$ per group) received injections of 1 μg IL-12 in chitosan solution (chitosan/IL-12) i.t., adjacent to the tumor or contralaterally on days 7, 14, 21 and 28. Mice receiving DPBS served as controls. For antitumor studies investigating the depot effect of chitosan, tumor-bearing mice ($n = 8$ per group) were treated i.t. with chitosan/IL-12 on days 7, 14, 21 and 28 or with equal microdoses of IL-12 (0.2 μg) alone on days 7–11, 14–18, 21–25 and 28–32. A third cohort of mice received decreasing doses of IL-12 alone to approximate the amount of IL-12 in the tumor following a single injection with chitosan/IL-12 as shown in our previous study [22]. Specifically, mice received 1 μg, 0.25 μg, 0.12 μg, 0.08 μg and 0.05 μg on days 7, 8, 9, 10 and 11, respectively. The same dose schedule was repeated starting on days 14, 21 and 28. Mice receiving DPBS served as controls. Tumor volumes were calculated using the modified ellipsoidal formula: tumor volume = ½ × length × width^2.

2.5. Immunophenotyping studies

C57BL/6J mice bearing established s.c. tumors were treated with DPBS, chitosan solution, 1 μg IL-12 or chitosan/IL-12 (1 μg) on days 9 and 14 after tumor implantation. On day 17, tumors, tumor-draining lymph nodes (TDLNs), i.e. the inguinal lymph node ipsilateral to the tumor, and spleens were harvested. Spleens and lymph nodes were mechanically disrupted with a syringe plunger and passed through a 70 μm nylon mesh strainer (BD Biosciences; Bedford, MA). Erythrocytes were lysed with ACK lysis buffer. Tumors were minced into 2 mm cubes and digested in Accumax at room temperature on an orbital shaker for 3 h. Viable cells were then collected on a histopaque gradient. Viable leukocytes from all tissues were quantified under trypan blue exclusion with an automated cell counter (Cellometer Auto T4; Nexcelom Bioscience; Lawrence, MA).

For flow cytometry studies, cells were washed twice with cold FACS buffer. FcγII and FcγIII receptors on leukocytes were blocked via incubation with 1 μg purified anti-mouse CD16/CD32 (clone: 2.4G2) (BD Biosciences; San Jose, CA) per 1×10^6 cells for 15 min on ice. Cell surface markers were stained with 1 μg fluorescence-labeled antibodies per 1×10^6 cells for 30 min on ice. A subset of cells were permeabilized with FACS buffer with 0.1% saponin and stained with fluorescence-labeled antibodies against intracellular markers for another 30 min on ice. Cells were then washed twice with cold FACS buffer prior to fixation in 1% paraformaldehyde, 2% glucose, and 5 mM sodium azide in PBS. Samples were rinsed twice and analyzed on a BD FACSCanto II (BD Biosciences; San Jose, CA) within 48 h. Data analyses were performed using FlowJo software v7.6.5 (Tree Star, Ashland, OR).

2.6. Statistical analysis

Differences in overall survival were analyzed using the logrank test. Differences in mean percentages of immune cell subsets were analyzed using Student's *t*-test with unpaired samples. *P*-values and hazard ratios were computed using GraphPad Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA).

3. Results

3.1. Effect of chitosan/IL-12 injection location

To determine if the location of chitosan/IL-12 injection relative to tumor impacts antitumor activity, mice bearing established s.c. MC38 tumors received weekly doses of chitosan/IL-12 injected s.c. adjacent to the tumor, s.c. contralateral to the tumor or i.t. The

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