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Design principles for cytokine-neutralizing gels: Cross-linking effects

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Abstract

Constructs composed of cytokine-neutralizing antibodies conjugated to high-molecular-weight hyaluronic acid have been shown to be effective at controlling inflammatory responses in vivo. A critical question in the development of this new class of biomaterial is whether crosslinked conjugates have similar anti-inflammatory effects, which would open up a broad range of tissue engineering applications in which the material would have intrinsic inflammation-controlling function. To test this, high-molecular-weight hyaluronic acid was conjugated with monoclonal antibodies to the pro-inflammatory cytokines interleukin-1 β and tumor necrosis factor- α in two forms of the material: viscous, non-crosslinked polymer–antibody conjugates and crosslinked, elastomeric polymer–antibody conjugates. The cytokine affinities of both constructs were validated using molecular characterization methods, and the biological activities were tested through subcutaneous implantation in Sprague–Dawley rats. In vitro, both forms of these constructs are capable of binding cytokines, but in vivo only the non-crosslinked polymer significantly reduces markers of acute inflammation compared to controls that lack the antibodies. We propose that these materials function by retarding cytokine diffusion, with the non-crosslinked polymers being capable of retarding the diffusion of cytokines in the extracellular matrix and preventing engagement with receptors. In contrast, crosslinked materials have long diffusion lengths into the gel compared with those between cells on the surface of the material, which may make them ineffective at sequestering pro-inflammatory cytokines on biologically relevant

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Appendix A. Figures with essential colour discrimination Certain figures in this article, particularly Figures 2, 3, 4, 5 and 7, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2010.06.029.

Appendix B. Supplementary data Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2010.06.029.

timescales. These results suggest an important design principle for preparing cytokine-regulating materials based on consideration of transport phenomena.

Keywords

Antibody; Biomimetic material; Pro-inflammatory cytokines; Hyaluronic acid; Inflammation

1. Introduction

Native inflammatory responses often dominate the effects of biomaterials that are intended to guide tissue repair [1]. These responses are characterized by invasion of macrophages, which initiate a host defense program involving production of reactive oxygen species and matrix-degrading enzymes. This program is driven by the production of high levels of inflammatory mediators, such as cytokines and chemokines [2], creating a self-perpetuating inflammatory microenvironment in which cells produce and respond to these mediators. Developing biomaterial strategies for controlling inflammation by specifically neutralizing these mediators could allow for reductions in deleterious host responses to implants, such as development of a chronic inflammatory state leading to the formation of giant cells [3].

In previous work, we demonstrated that application of constructs composed of cytokine-neutralizing antibodies and high-molecular-weight hyaluronic acid (HA) significantly decreased inflammatory responses in a rat incisional wound model [4]. Application to the wound site of these constructs, containing monoclonal antibodies against interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α), two central mediators of early inflammation [5–7], resulted in a 30% reduction in the number of macrophages observed at the wound site and concomitant reductions in the numbers expressing markers of classical activation (M1 phenotype) and alternative activation (M2 phenotype). This suggests that these materials are dampening the inflammatory response through neutralization of intercellular signaling proteins.

Cytokine-neutralizing gels represent a biomaterials approach to controlling inflammation locally [8]. Native extracellular matrix regulates the activities of a broad range of soluble signaling proteins through specific and non-specific binding interactions [9,10], with examples including binding of transforming growth factor- β 1 by fibronectin [11] and heparin binding of vascular endothelial growth factor-165 [12]. Biomaterials capable of similar binding function could indirectly control cellular activities by regulating which soluble signaling proteins are available to interact with cell receptors. Controlling inflammatory mediators may not be a native function of the ECM because these mediators have an evolved function that is intended to provide immediate signaling, but engineered ECM could certainly provide such biological function [13]. However, while viscous solutions of polymer–antibody conjugate would be useful for certain dermatological or injectable applications, crosslinked polymer–antibody hydrogels that locally control inflammation could find broader application in regenerative medicine.

Anseth and co-workers described an approach in which anti-TNF- α peptides were conjugated to crosslinked hydrogels and shown to protect therapeutic stem cells from

inflammatory mediators [14]. They demonstrated in vitro that cell survival in the gels improved when the cells were cultured in the presence of TNF- α compared to cells immobilized in gels that lacked this peptide, suggesting that the conjugated peptides are still capable of binding TNF- α diffusing through the gel. A different question would be whether hydrogels that neutralized pro-inflammatory cytokines are capable of influencing native inflammatory responses in surrounding tissues.

In this work, we covalently attached monoclonal antibodies (mAb) to high-molecular-weight HA and compared these to cross-linked HA and mAb polymerized via pendant methacrylate groups. While HA with molecular weight greater than 1 MDa is soluble in aqueous solutions, it forms a highly viscous solution at mass fractions of less than 1% [15] and can be easily modified to produce crosslinked hydrogels [16,17]. We verified binding affinity of the cytokine-antibodies and tested the effects of these constructs in a rat incisional wound model.

2. Materials and methods

2.1. Materials

HA ($\sim 1.6 \times 10^6$ g mol⁻¹), glycidyl methacrylate, *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and 4-(dimethylamino)-pyridine (4-DMAP) were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Acryloyl-poly(ethylene glycol)-*N*-hydroxysuccinimide (ACRL-PEG-NHS, 3.4 kDa) was purchased from Nektar Therapeutics (San Carlos, CA). The oligopeptide sequence GRGDS was synthesized according to standard Fmoc solid-phase peptide synthesis. The Fmoc-protected amino acids dicyclohexylcarbodiimide and 1-hydroxybenzotriazole were purchased from EMD Chemicals Inc. (San Diego, CA) and used as received. Anti-hIL-1 β from purified mouse monoclonal IgG₁ was purchased from R&D Systems, Inc. (Minneapolis, MN) with a reported binding affinity for human IL-1 β of 120 pM. Monoclonal anti-human IL-1 β antibody and IL-1 β (IL-1F2) were also purchased from R&D Systems, Inc. Rat anti-mouse IL-1 β and TNF- α monoclonal antibodies (R&D Systems) were used for in vivo studies, with rat-mouse cross-reactivity values of 95% and 90%, respectively, reported by the manufacturer. Reagents were reconstituted and stored according to the manufacturer's instructions.

2.2. Non-crosslinked HA-mAb preparation

The first step reaction consisted in the activation of the carboxylic acid from the glucuronic acid moiety. The HA-active ester intermediate was subsequently used as a precursor for the coupling reaction with anti-hIL-1 β . HA (10 mg, 6.25 nmol) was dissolved in phosphate-buffered saline (PBS; pH $\sim$ 7.4) (2 ml). EDC (120 μ g, 625 nmol), sulfo-NHS (217 μ g, 1 μ mol) and 4-DMAP (10 μ g) were added as solids to the HA solution and allowed to dissolve and react overnight before adding anti-hIL-1 β . The HA-NHS active ester is a versatile precursor for bioconjugation with primary amines. Anti-IL-1 β was added alone or with anti-TNF- α (0.3 mg each) to the activated HA solution. The reaction proceeded at room temperature for 17 h. The solution was precipitated in a saturated solution of ammonium sulfate, and the product was recovered by centrifugation. The pellet was reconstituted in

PBS and followed by dialysis (Nest Group, Southborough, MA) against pure PBS for 4 h (MW cut-off 300 kDa).

2.2.1. Crosslinked HA–mAb preparation—The conjugation of HA, GRGDS and mAb with polymerizable methacrylate or acrylate groups followed established procedures [16]. Briefly, we prepared HA–glycidyl methacrylate (GM) polymers by treating a 0.5% w/v solution of fermentation-derived HA ($\sim 1.6 \times 10^6$ Da) in PBS and dimethylformamide with a 50-fold molar excess of GM in the presence of excess triethylamine. The reactions were carried out at 25 °C for 6 days and purified via precipitation and dialysis against PBS. Gels having a degree of methacrylation of 23% and a HAGM mass fraction of 10% were prepared with methacrylate-functionalized HA, GRGDS and mAb. Acrylate-functionalized GRGDS and mAb were prepared by reacting with stoichiometric amounts of ACRL-PEG-NHS. Crosslinked gels composed of 10% HA by mass, 5 mM RGD-PEG-acrylate and 100 mg ml⁻¹ mAb-PEG-acrylate.

2.3. Polyacrylamide gel electrophoresis

Ten microliters of 4% acrylamide/bis-acrylamide solution in 1% TBE buffer was prepared from 40% acrylamide/bis-acrylamide solution (Sigma, MO) and 10 × TBE buffer (Promega, Madison, WI). The solution was mixed on a stir plate for 10 min, then 50 µl of 10% (w/v) ammonium persulfate and 4 µl of *N,N,N',N'*-tetramethylethylenediamine (Sigma–Aldrich) were added. The solution was mixed well and injected into glass plates (Bio-Rad, Hercules, CA). After 30 min to 1 h, the gel hardened. Then 5 µl of each of the standards consisting of 0.1%, 0.05% and 0.025% HA in the gel were loaded at two different concentrations using 1× and 0.1× stock solutions, and 125 V used to electrophorese the gel for 5 h.

2.4. Hyaluronan quantification

Gels were stained in 0.5% Alcian Blue (Sigma–Aldrich) with 3% acetic acid for 45 min followed by destaining with 3% acetic acid overnight. Gel images were taken and quantitatively analyzed using Fujifilm LAS-3000 and MultiGauge image analysis software.

2.5. Fluorescence immunosorbent assay

An Immuno 96 MicroWell Plate (NUNC/Thermo-Fisher, Rochester, NY) was first incubated with 50 µl of 1 µg ml⁻¹ of rabbit anti-rat IgG (Jackson Laboratories, Bar Harbor, ME) in PBS in each well at 4 °C overnight. The solution was removed and the plate was washed three times with detergent followed by incubation at 37 °C for 1 h in 200 µl of the blocking buffer, which contained 0.25% bovine serum albumin (BSA), 0.05% Tween and 1 mM ethylenediaminetetraacetic acid in 1× PBS. The plate was subsequently washed. IL-1 antibody was dissolved in carbonate buffer, and standards were prepared using rat whole IgG (Jackson Laboratories) in triplicates. Fifty microliters of each solution was loaded into designated wells followed by 1 h of incubation with shaking at room temperature. The solutions were discarded and each well was washed in detergent three times with 10 min of incubation in between. Goat anti-rat IgG (2 µg ml⁻¹) was conjugated with Alexa 488 (Invitrogen, Carlsbad, CA), prepared in carbonate buffer. Fifty microliters of this solution was loaded into each well followed by 1 h incubation with shaking in the dark at room temperature. Wells were washed again three times with detergent for 10 min in between and

the plates preserved with PBS. The plate was read and analyzed by SAFIRE microplate reader with excitation at 488 nm and emission at 520 nm.

2.6. Binding affinity measurements

The Octet system (ForteBio Corp., Menlo Park, CA) was utilized to measure HA-mAb binding affinity for IL-1 β . The Octet measures the reflection coefficient as broadband visible light propagates to the end of a fiber optic. Changes in the refractive index at the fiber optic-solution interface result in a wavelength-dependent shift in the maximum of the reflection coefficient. Association and dissociation curves are fitted to equations of the following form:

$$R(t) = R_0 + \Delta R \{1 - \exp[-k_{on}(t - t_{on})]\} \quad (1)$$

$$R(t) = R_0 + \Delta R \exp[-k_{off}(t - t_{off})] \quad (2)$$

where $R(t)$ is the reflection coefficient at time t , R_0 is the baseline value of the reflection coefficient, ΔR is the total change in response, k_{on} is the association rate constant, t_{on} is the time at which the sensor is placed in solution containing the analyte, k_{off} is the dissociation rate constant and t_{off} is the time at which the sensor is placed in pure buffer solution. The values for k_{on} and k_{off} are determined from curve fitting, and their ratio provides a measurement of K_D . Mouse anti-human IL-1 β monoclonal antibody and HA-conjugated antibody were first biotinylated using EZ-link NHS-LC-LC-Biotin (Thermo Scientific) with a 1:1 ratio. The biotinylated antibody was then dialyzed for 8 h in 4C with several changes of PBS. Streptavidin sensor tips were hydrated in kinetics buffer (0.02% Tween 20, 1 mg ml⁻¹ BSA, 10 mM PBS and 0.05% sodium azide, supplied by ForteBio) for at least 5 min before the experiment. All the samples were diluted in buffer. Biotinylated mouse anti-human IL-1 β monoclonal antibody was diluted to 10 μ g ml⁻¹ and the HA-mAb sample was diluted to the equivalent concentration of antibody. Human IL-1 β was diluted to the desired concentration in kinetics buffer. The experimental setup sequence is as follows: kinetics buffer 5 min (baseline), antibody or HA-mAb solution 15 min (loading), kinetics buffer 5 min (wash), kinetics buffer 5 min (baseline), IL-1 β solution 30 min (association) and kinetics buffer 60 min (dissociation). The results were analyzed by the ForteBio analysis program that generated the best-fit binding isotherm and the association rate k_{on} and dissociation rate k_{off} were calculated from the isotherm.

2.6.1. Diffusion measurements—The affinity of anti-IL-1 β incorporated into crosslinked HAGM gels was verified by measuring the time-dependent concentration of IL-1 β in solutions placed above the gels in 96-well plates. One-dimensional diffusion measurements can be used to estimate the concentration of binding groups using a reaction-diffusion formalism [18]. Briefly, if the chemical potential of IL-1 β is assumed to be zero when bound by the mAb, the equilibrium constant for the reaction

can be used to predict the equilibrium concentration of IL-1 β in the solution using the measured K_D of the mAb. Because the instantaneous concentration gradient drives the

cytokine flux from the solution into the gel, the time-dependent concentration is predicted to decay exponentially with time until equilibrium is reached [18]. The concentration of IL-1 β at each time point was measured using an enzyme-linked immunosorbent assay (ELISA) following similar protocols to those described above.

2.6.2. In vivo studies—Subcutaneous implantation of biomaterials in healthy adult Sprague–Dawley rats was performed to evaluate the effect of non-crosslinked and crosslinked HA–mAb materials upon the local acute inflammatory response of the host. All animal experiments were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals and the University of Pittsburgh Institutional Animal Care and Use Committee (protocol number 08,07,912). Four separate 1 cm incisions were made on the backs of shaved, anesthetized animals. The underlying fascia was damaged with a scalpel to separate the skin and the underlying muscle. To accentuate the damage, the skin was scraped five times from underneath. The site was then treated with saline, non-crosslinked HA–RGD, non-crosslinked HA–RGD–mAb, crosslinked HA–RGD or crosslinked HA–RGD–mAb, then sutured shut. After 4 days, the animals were sacrificed and the sites were collected for histological analysis.

2.6.3. Immunohistochemistry—The harvested skin tissues were fixed in formalin and embedded in paraffin prior to being cut into 6 μ m thick sections and mounted on glass slides. The specimens were deparaffinized with xylene followed by exposure to graded series of ethanol solutions (100–70%). Sections were stained with Masson's trichrome for morphological assessment. Immunohistochemistry was performed on consecutive 6 μ m sections of each specimen in order to assess the macrophage phenotype surrounding the incision injury site. Following deparaffinization, the slides were placed in citrate antigen retrieval buffer (10 mM citric acid monohydrate, pH 6.0, Spectrum), which was then brought to a boil (95–100 °C) for 20 min. The buffer was allowed to cool and the slides were then washed twice in Tris-buffered saline/Tween 20 (both from Sigma) solution (pH 7.4) and twice in PBS. The sections were incubated in 1% BSA for 1 h at room temperature in a humidified chamber to inhibit non-specific binding of the primary antibody. Following incubation in blocking serum, the sections were incubated in primary antibody in a humidified chamber at 4 °C overnight. Each tissue specimen was exposed to antibodies to a pan-macrophage marker (CD68), an M1 phenotype marker (CCR7) and an M2 phenotype marker (CD163). Following the overnight incubation, the slides were washed three times in PBS. Sections were then incubated in a solution of 3% H₂O₂ in methanol for 30 min at room temperature to quench endogenous peroxidase activity. Following H₂O₂ treatment, the slides were washed three times in PBS prior to incubation in secondary antibody for 30 min in a humidified chamber maintained at 37 °C, then subjected to three more washes in PBS. The sections were then incubated in Vectastain ABC (Vector, CA) reagent for 30 min in a humidified 37 °C chamber, rinsed three times in PBS and incubated in 4% diaminobenzadine substrate solution (Vector, Burlingame, CA) at room temperature. The slides were rinsed in water to stop the development of the diaminobenzadine substrate and counterstained using Harris hematoxylin stain (Thermo Electron Corporation-Shandon, Pittsburgh, PA). The slides were then dehydrated using the reverse of the deparaffinization treatment and each sample had a coverslip placed on top. Each PBS rinse in the protocol was

for 3 min at room temperature, with occasional agitation. The primary antibodies used were mouse anti-rat CD68 (Serotec, Raleigh, NC) at a dilution of 1:50, rabbit anti-CCR7 (Epitomics, Burlingame, CA) at a dilution of 1:250 and mouse anti-rat CD163 (Serotec) at a dilution of 1:50. The secondary antibodies used were biotinylated horse anti-mouse IgG (Vector, CD68 and CD163) at a dilution of 1:50 and goat anti-rabbit IgG (CCR7, Vector) at a dilution of 1:100. All antibodies were diluted in filtered PBS (pH 7.4).

2.6.4. Immunohistochemical analysis—The immunostained slides were imaged using a Nikon e600 microscope. The images were then evaluated quantitatively in a blinded fashion by three independent investigators (N.M.D., J.M.P. and K.A.). Quantitative analysis was performed by counting the number of immunopositive cells in three matched microscope fields at 20× magnification. The average and standard deviation of the number of positively stained cells were used to calculate the final results.

3. Results

3.1. Preparation of non-crosslinked HA–mAb and crosslinked HA–mAb

To prepare non-crosslinked HA–mAb for in vivo testing, HA was coupled with anti-TNF- α and anti-IL-1 β using carbodiimide coupling to form a peptide bond between an activated carboxylate group on the HA and an amine on the mAb following reported methods [4]. As measured using polyacrylamide gel electrophoresis and a fluorescent immunosorbent assay similar to ELISA, 14% of the HA chains were functionalized with mAb. Crosslinked HA–mAb hydrogels were prepared by first reacting both HA and mAb with glycidyl methacrylate to provide a site for photopolymerization of both biomolecules. Hydrogels were prepared with nominal ratio of HA:mAb equal to 1:7, similar to the non-crosslinked HA–mAb samples. Preparation schemes for both non-crosslinked and cross-linked HA–mAb are shown in Fig. 1.

3.2. Cytokine binding affinity of HA–mAb conjugate

The binding constant of the non-crosslinked HA–(anti-IL-1 β) conjugate with IL-1 β was measured and compared with that of the non-conjugated anti-IL-1 β . Sensor tips functionalized with streptavidin were used to mount antibody onto the sensor surface while preserving the accessibility of the IL-1 β -binding domains. The rise of the signal in Fig. 2 demonstrated the change in refractive index at the sensor–solution interface, which indicated that the mAb and HA–mAb conjugates were absorbed onto the sensor. Although the interaction between streptavidin and biotin is very strong, a slight drifting baseline was still observed. Therefore, association and dissociation isotherms were corrected with corresponding baselines to obtain accurate results.

In Table 1 are shown the association and dissociation data corrected for baseline variations and the best-fit isotherms. The K_D of unmodified anti-IL-1 β mAb was measured to be 118 ± 17.4 pM and that of HA–anti-IL-1 β conjugate was 40.1 ± 4.8 pM. This difference was attributed to similar association curves but a slightly slower dissociation constant of IL-1 β on the HA–mAb-coated sensor. The presence of the HA matrix on the sensor tip could prevent IL-1 β from freely dissociating from the sensor surface. While the exact nature of

this asymmetry is uncertain, it can be concluded that HA-mAb retains a significant affinity for IL-1 β .

3.3. Cytokine binding affinity of crosslinked HA-mAb conjugate

One-dimensional diffusion experiments were performed to test whether antibodies incorporated into crosslinked HA matrices was still capable of binding IL-1 β . The time-dependent concentration of IL-1 β solutions placed in contact with crosslinked HA gels having the equal volumes as the solution overhead and containing anti-IL-1 β with concentrations of 0, 2.5 or 330 $\mu\text{g ml}^{-1}$, shown schematically in Fig. 3. The starting concentration of IL-1 β in the solution was arbitrarily chosen to be 1000 ng ml^{-1} , and monotonic decreases in cytokine solution concentration were observed as a function of time, as shown in Fig. 4. The data were fitted to a function of the form.

$$y(t) = y_0 + A \exp(-t/\tau) \quad (4)$$

where $y(t)$ is the instantaneous IL-1 β concentration, y_0 and A are constants with units of nanograms per milliliter, t is time in units of hours and τ is the time constant that describes the temporal decay of the IL-1 β concentration in top solution. The results of the fits are shown in Table 2.

The final concentration in the solution over the HA gel with no antibody was found to be 372 ng ml^{-1} (theoretical prediction: 500 ng ml^{-1}). The gel containing 2.5 $\mu\text{g ml}^{-1}$ anti-IL-1 β had a final solution value of 298 ng ml^{-1} (theoretical prediction: 250 ng ml^{-1}), while that containing 330 $\mu\text{g ml}^{-1}$ anti-IL-1 β went to a final measured solution concentration of less than 20 ng ml^{-1} (theoretical prediction: 1 ng ml^{-1}), suggesting that crosslinked gels containing mAb are able to sequester pro-inflammatory cytokines effectively. The reasonable agreement between the theoretical and measured values suggests that the mAb retain their binding affinities in the gels, consistent with the results reported by Lin et al. [14] obtained using fluorescence resonance energy transfer measurements.

The temporal profile of the IL-1 β concentration decayed exponentially, with time constants of 59.6 h for 0 $\mu\text{g ml}^{-1}$ anti-IL-1 β , 19.8 h for 2.5 $\mu\text{g ml}^{-1}$ and 30.8 h for 330 $\mu\text{g ml}^{-1}$. The non-monotonic dependence of the time constants on mAb concentration may be due to underlying multi-modal transport phenomena, such as convective transport upon addition of the cytokine solution over the gel that could have depleted the IL-1 β solution concentrations initially. However, the plateau values appeared to fit well to a single exponential function, suggesting the complex behaviors at early times damped quickly. The long equilibration times ultimately characterized the transport of cytokine into the hydrogels, and these had their basis in slow protein diffusion in solution, also similar to the long equilibration times measured by Lin et al. [14]. To test whether this was too slow to be effective at modulating inflammatory responses and to compare the data with the non-crosslinked material, which presumably would not have similar transport limitations in binding cytokines in vivo, subcutaneous implantation studies in rats were performed.

3.4. In vivo studies

Subcutaneous implantation was performed using non-cross-linked and crosslinked HA materials to compare the biological activities of these materials as a function of antibody functionalization and to compare them with saline-treated sites. The RGD adhesion peptide was incorporated into the formulations to make them cell-adhesive, so the four compositions tested were HA–RGD, HA–RGD–mAb, crosslinked HA–RGD and crosslinked HA–RGD–mAb, in which mAb formulations contained both anti-IL-1 β and anti-TNF- α .

In Fig. 5A is shown a representative section of the sites treated with HA–RGD stained with hematoxylin and eosin (H&E) and imaged at 4 $\times$ magnification, with a 10 $\times$ image of the site shown in the inset. The location of the original incision is identified with a black line, and a clean separation between the dermis and the underlying muscle was still observed at 4 days with robust infiltration of mononuclear cells at the edges of the tissue and the layer of damaged fascia. Narrow layers of porous tissue are observed at the bottom left of the 4 $\times$ image, which we attributed to damage caused when the tissue was separated prior to treatment.

Fig. 5B shows that the site treated with HA–RGD–mAb conjugated with mAb against both IL-1 β and TNF- α had significant reductions in the number of mononuclear cells observed at the tissue edges, suggesting cytokine neutralization had an inhibitory effect on cell recruitment. This effect is observed both around the original incision, identified with a black line, as well as in the region where the fascia was damaged. Furthermore, the gross histological features of this site were qualitatively different from the sites treated with saline or HA–RGD. A significant amount of local tissue edema was present when the site was collected, and the layers of tissue appeared to be more connected, suggesting that the early phases of wound healing had been inhibited. Interestingly, we did not see similar changes in the histological features at the sites treated with HA–RGD–mAb, where only IL-1 β was neutralized. This is consistent with the understanding of the complementary roles played by IL-1 β and TNF- α in acute inflammation [1]. Future studies will involve assays of cytokine production and other inflammatory markers that may be more sensitive to changes in signaling of IL-1 β .

These results were compared with sites treated using cross-linked versions of the same formulations of HA–RGD and HA–RGD–mAb. Shown in Fig. 5C is the site that was treated with cross-linked HA–RGD. The gels did not adhere strongly to the tissue and separated during histological processing. In these samples, very few fragments of the original gel were observed in the tissue section, and one that was embedded in growing tissue is marked with an asterisk. The original location of the gel following implantation is also identified with an asterisk. The presence of the gel, which was a disk having diameter of 8 mm and a height of 4 mm, also prevented simultaneous observation of the upper and lower faces of the separated tissue. The upper tissue had a robust invasion of mononuclear cells similar to that observed in the saline-treated sites performed in our previous work on HA–mAb conjugate [4].

Tissue sections from the sites treated with crosslinked HA–RGD–mAb had similar tissue responses, as shown in Fig. 5D. The gel in this site fractured, and fragments can be seen at

the bottom of the inset in the H&E section, each identified with an asterisk. Mononuclear cells have migrated to the surface of the tissue and do not appear to be inhibited by the presence of cytokine-binding antibodies within the material. This insensitivity to the presence of cytokine-neutralizing antibodies suggests that the material geometry plays a significant role in determining the ability to regulate soluble signaling proteins.

Immunohistochemical staining was performed to assess the effects of mAb-functionalized materials on the state of inflammation. We measured the number of macrophages and the fraction expressing M1 and M2 phenotypic markers: CD68 was used as a pan-macrophage marker, CCR7 was used to identify macrophages expressing M1 markers and CD163 was used to identify macrophages expressing M2 markers [19]. As discussed in our preliminary work on cytokine-neutralizing gels [4], the average number of cells that stained positively for CD68, CD163 and CCR7 were 578.8, 433 306.5 respectively in the saline-treated site. Saline treatment was used as a negative control to establish a baseline for an incisional injury. In Fig. 6 are shown the average number of cells that stained positively for each marker in the current study. The injury sites treated with HA-RGD had significantly higher numbers of macrophages ($p < 0.05$) as well as a greater number expressing the M1 phenotype marker ($p < 0.05$) than those treated with HA-RGD-mAb, suggesting that antibody functionalization of non-crosslinked materials modulated the inflammatory response. The macrophage profile in the injury site treated with crosslinked HA-RGD gel was significantly different from that in non-crosslinked HA-RGD conjugate. Greater numbers of M1 macrophages were observed in non-crosslinked HA-RGD conjugate treated sites than in the crosslinked HA-RGD gel treated site, yet the numbers of M2 macrophages remained similar. This observation could be interpreted as suggesting that fewer HA degradation products were released from crosslinked gel to act as damage-associated molecular patterns that would activate an immune response [20]. Furthermore, the injury sites treated with crosslinked HA-RGD and crosslinked HA-RGD-mAb had similar profiles of macrophages, indicating that the crosslinked HA-RGD-mAb gel has limited ability to reduce inflammatory response. This is probably due to the slow diffusive property of the proteins shown above by the in vitro diffusion experiment. Representative images of CD68 staining are available in Supplementary material.

4. Discussion

Conjugates of HA and mAb were prepared to impart specific binding of pro-inflammatory cytokines by materials and modulate inflammatory responses in vivo. While both non-crosslinked and crosslinked materials demonstrated binding of cytokines in vitro, only antibody functionalization of the non-crosslinked material appeared to have a significant impact on the inflammatory response in vivo, although the intrinsic biological activities of non-crosslinked HA clearly impacted on the responses. Our results indicate that material geometry has a significant role in determining biological activity.

We propose that there are two characteristic length scales that determine biological activity of these materials: cell-cell distance and cell-antibody distance. The material geometry determines the latter characteristic length scale, and if the cell-cell distance is short relative to cell-antibody distance, cytokine diffusion between cells becomes the dominant outcome,

as observed in the crosslinked HA–RGD–mAb gels. However, if the cell–antibody distance is comparable to or less than the cell–cell distance, as is likely the case for the non-crosslinked HA–RGD–mAb material, then a high-affinity antibody can retard cytokine diffusion and the inflammatory response is inhibited. This is consistent with mathematical models of cytokine signaling [21], even when the antibody affinity for the cytokines (~100 pM) is weaker than the binding affinity of the receptor (~10 pM) [22]. These limiting cases are depicted schematically in Fig. 7. Furthermore, this model predicts that the anti-inflammatory effects of crosslinked, cytokine-neutralizing gels are independent of mesh size, as long as cytokines are able to diffuse freely in the gel and cells are restricted to the gel exterior. However, this prediction needs to be tested.

5. Conclusions

Monoclonal antibodies against pro-inflammatory cytokines demonstrated specific affinities for their cognate antigen following covalent incorporation in hyaluronic acid constructs. Non-cross-linked materials had similar equilibrium binding constants as unconjugated antibodies while antibody-functionalized crosslinked materials were able to effectively sequester pro-inflammatory cytokines in vitro. However, results from in vivo testing suggest that only the non-crosslinked hyaluronic acid–antibody conjugates were effective at inhibiting inflammatory responses. These results indicate that antibody functionalization is not a viable strategy for imparting anti-inflammatory function in tissue engineering hydrogels. To design materials capable of regulating the activities of soluble signaling proteins, we propose a model in which the material must be integrated into the tissue microenvironment in order to compete for signaling molecules. In this model, material geometry becomes a proxy for considerations of cytokine transport phenomena. This approach could be generalized to develop a new class of biomaterials capable of indirectly guiding cellular responses by regulating the activities of soluble signaling proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Reaction schemes showing the preparation of (A) non-crosslinked HA-mAb conjugates and (B) crosslinked HA-mAb conjugates.

Fig. 2. Adsorption and desorption data from optical biosensor measurement of (A) anti-IL-1 β /IL-1 β interactions and (B) HA-anti-IL-1 β /IL-1 β interactions. The plots at the right show the residuals from the fits to kinetic equations described in Section 2.

Fig. 3.
Schematic representation of the one-dimensional diffusion experiments performed to determine affinity of crosslinked HA-(anti-IL-1 β) for IL-1 β .

Fig. 4. Concentration of IL-1 β as a function of time for IL-1 β solutions over crosslinked HA-(anti-IL-1 β) gels.

Fig. 5.

Histological images (4× and 10×) from injury sites treated with (A) HA-RGD, (B) HA-RGD-mAb, (C) HA-RGD gel and (D) HA-RGD-mAb gel. (A) Robust infiltration of mononuclear cells (indicated by →) along the incision injury (indicated by —) and at the edge of the tissue where the fascia was damaged. The separation of the skin and underlying muscle is still visible. (B) Moderate infiltration of mononuclear cells was observed in this injury site, and the fascia appears to be loosely connected. (C) HA-RGD gel was implanted at the location indicated by an asterisk (*), and a fragment of the gel is shown in the image. Robust infiltration of the mononuclear cells can be observed around the gel. (D) Several fragments of the implanted HA-RGD-mAb gel were shown, as identified with an asterisk (*), and a layer of mononuclear cells was apparent at the interface between the gel and the tissue.

Fig. 6.

Quantification of immunohistochemical staining of tissue sites treated as noted. Bars shaded with vertical lines were stained for the pan-macrophage marker CD68, bars with diagonal shading were stained for the macrophage M2 phenotype marker CD163 and bars shaded solid black were stained for the macrophage M1 marker CCR7. Select p -values are written above the double-headed arrows connecting results that have significantly different values.

Fig. 7.

Schematic representation of how non-crosslinked HA–mAb constructs are hypothesized to interrupt cytokine signaling (left) while crosslinked HA–mAb constructs do not (right). HA strands are represented as lines, mAb as Y-shapes, cytokines as triangles and cell receptors as trapezoidal shapes with complementary binding pockets for cytokines.

Table 1

Association and dissociation rate constants and calculated equilibrium binding constants comparing unmodified anti-IL-1 β and non-crosslinked HA-anti-IL-1 β affinities for IL-1 β .

	k_{on} (10^5 M s^{-1})	k_{off} (10^{-5} s^{-1})	K_D (10^{-10} M)
Anti-IL- β mAb	6.548 ± 0.344	7.906 ± 1.506	1.180 ± 0.174
HA-anti-IL-1 β mAb	6.247 ± 0.228	2.505 ± 0.320	0.401 ± 0.048

Table 2

Results from fitting data on IL-1 β diffusion into crosslinked HA-anti-IL-1 β gels containing varying concentrations of anti-IL-1 β .

Anti-IL-1 β Conc. ($\mu\text{g/ml}$)	y_0 (ng ml^{-1})	A (ng ml^{-1})	τ (hrs)	R^2
0	372	614	59.6	0.982
16	298	680	19.8	0.892
330	17	767	30.8	0.967