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Spatiotemporal effects of a controlled-release anti-inflammatory drug on the cellular dynamics of host response

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Abstract

In general, biomaterials induce a non-specific host response when implanted in the body. This reaction has the potential to interfere with the function of the implanted materials. One method for controlling the host response is through local, controlled release of anti-inflammatory agents. Herein, we investigate the spatial and temporal effects of an anti-inflammatory drug on the cellular dynamics of the innate immune response to subcutaneously implanted poly(lactic-co-glycolic) microparticles. Noninvasive fluorescence imaging was used to investigate the influence of dexamethasone drug loading and release kinetics on the local and systemic inhibition of inflammatory cellular activities. Temporal monitoring of host response showed that inhibition of inflammatory proteases in the early phase was correlated with decreased cellular infiltration in the later phase of the foreign body response. We believe that using controlled-release anti-inflammatory platforms to modulate early cellular dynamics will be useful in reducing the foreign body response to implanted biomaterials and medical devices.

Keywords

foreign body response; controlled drug release; dexamethasone; cellular dynamics; inflammatory proteases

Introduction

One major challenge to clinical application of biomaterials and medical devices is their potential to induce a non-specific host response[1–8]. This reaction involves the recruitment

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of early innate immune cells such as neutrophils and macrophages, followed by fibroblasts which deposit collagen to form a fibrous capsule surrounding the implanted object[1, 8–11]. Fibrotic cell layers can hinder electrical[12] or chemical communications and prevent transport of analytes[13–15] and nutrients, thus leading to the eventual failure of many implantable medical devices such as glucose sensors[3, 4, 16], neural probes[17], immunoisolated pancreatic islets[18–20] and biodegradable polymeric stents[5].

The incorporation of controlled-release delivery systems of anti-inflammatory drugs into medical devices has been proposed to mitigate host response and improve device durability[21–25]. This approach has shown promise in a number of clinical applications. For example, controlled elution of steroids from pace-maker leads has reduced fibrosis formation and enhanced long-term electrical communication between the leads and surrounding cardiac tissue[12]. However, similar attempts to improve the performance of other medical devices such as implanted glucose sensors[26] and immunoisolated islets for diabetes therapy have proven challenging[1]. There remains a substantial need to better understand the immunomodulatory effects of anti-inflammatory drugs on the host-tissue biology at the implant site[21]. Such knowledge can lead to better design of controlled-release drug delivery systems to improve the biocompatibility of implanted medical devices.

Researchers developing controlled-release drug formulations to mitigate host response have largely focused on decreasing the number of inflammatory cells infiltrating the host-device interface. Hickey *et al.* designed a mixed microsphere system containing dexamethasone, a steroidal anti-inflammatory drug, to achieve zero-order *in vitro* release kinetics and to suppress tissue response to thread-induced injuries in rats for up to one month[27, 28]. Recent studies on a hydrogel composite containing dexamethasone-loaded poly(lactic-co-glycolic) (PLGA) particles also suggested that sustained release of this drug may minimize the inflammatory reactions at the tissue-material interface[29–31]. While these studies have provided invaluable information, they only addressed the effects of these drug delivery systems via *ex vivo* analysis of the cell types, quantity and distribution in excised tissues. However, various factors in the design of controlled-release formulations such as drug selection, drug loading, particle sizes and corresponding release kinetics can dynamically affect a range of biological activities in the host response. The presence of anti-inflammatory drugs may alter not only the quantity and variety of immune cells recruited but also the kinetics of cellular activities such as the secretion of inflammatory enzymes or cell signaling pathways[32, 33]. *In vivo* cellular secretory products might affect the degradation rate of the polymeric matrix[34–36] used to encapsulate drugs, and are partly responsible for the discrepancy between *in vitro* and *in vivo* release kinetics[37]. Therefore, we hypothesize that monitoring the spatial and temporal dynamics of enzymatic activity in the host response will offer new insight into the efficacy of controlled-release systems of anti-inflammatory drugs.

In this study, we examined the real-time effects of controlled-release anti-inflammatory therapeutics on the host response to subcutaneously implanted polymeric materials. Poly(lactic-co-glycolic) (PLGA 50/50) microparticles with and without dexamethasone were subcutaneously injected in a six-spot array on the dorsal side of immunocompetent mice. Monitoring the *in vivo* activity of cathepsins, a class of inflammatory proteases, by *noninvasive* fluorescent imaging revealed that microparticles with low drug loading (1.3wt

%) locally inhibited these enzymes, while high drug loading (26wt%) formulation resulted in systemic immunosuppression. The low dexamethasone loading (1.3wt%) was sufficient to attenuate the coverage of the implanted polymer by fibrotic cell layers. Temporal monitoring of the anti-inflammatory effect was carried out by *in vivo* imaging and *ex vivo* histological analysis.

2. Materials and Methods

2.1 Fabrication and characterization of PLGA microparticles

Microparticles with or without dexamethasone were prepared using a single-emulsion method [38] with biodegradable PLGA 50/50 (inherent viscosity of 0.95–1.20dl/g) from Lactel (Pelham, AL). Typically, a 5mL solution of PLGA and dexamethasone dissolved in dichloromethane, at concentrations of 40mg/ml and 2mg/ml respectively, was quickly added to a 25mL solution of 1% (w/v) polyvinyl alcohol and homogenized for 60s at 5000rpm (Silverson L4R, Silverson Machines Ltd., Cheshire, England). The resulting suspension was quickly decanted into 75mL of deionized water and stirred for 30s prior to rotary evaporation (Buchi Rotavap, Buchi, Switzerland) for 3min. The suspension was washed five times by centrifugation at 3000rpm for 3min. The particles were collected by filtration using 0.2µm filter, flash-frozen in liquid nitrogen, and lyophilized to dryness. Particle size distribution and morphology were examined by Scanning Electron Microscopy (JSM-6060, Jeol Ltd., Peabody, MA, USA). Fluorescence spectra of the PLGA polymer microparticles were collected by a Fluorolog-3 spectrofluorometer (Horiba Yvon Jobin, Edison, NJ, USA). The dexamethasone loading of all microparticles was determined by dissolving 2mg of microspheres in 1mL of acetonitrile and comparing the resulting UV absorbance at 234 nm to a standard curve of known concentrations of dexamethasone in acetonitrile.

2.2 In vitro drug release kinetics

The sample preparation and separation methods reported elsewhere were utilized to study the release of drug from microparticles[39]. Briefly, 3.5mg of dexamethasone-loaded PLGA microparticles were suspended in 1mL of 0.9% (w/v) NaCl solution in a 1.5mL centrifuge tube. The centrifuge tube was incubated at 37°C on a tilt-table (Ames Aliquot Mixer, Miles). At predetermined intervals, the tube was centrifuged at 12krpm for 5min using an Eppendorf 5424 microcentrifuge. The supernatant was collected and replaced with an equal volume of fresh 0.9 % (w/v) aqueous NaCl solution. After a release period of thirty days, the suspension of remaining particles was completely dissolved in acetonitrile overnight. The concentration of dexamethasone in all collected samples was quantified using UV absorbance at 234nm against a standard curve of known drug concentrations. The percentage of drug release at each time point was calculated by normalizing the cumulative amount of drug collected at each point with the total amount of drug initially encapsulated in the particles. The release kinetics reported for each particle formulation was obtained from the average of quadruplicate experiments.

2.3 Animal care

The animal protocol was approved by the local animal ethics committees at Massachusetts Institute of Technology (Committee on Animal Care) and Children's Hospital Boston

(Institutional Animal Care and Use Committee) prior to initiation of the study. Male SKH-1E mice at the age of 8–12 weeks were obtained from Charles River Laboratories (Wilmington, MA, USA). The mice were housed under standard conditions with a 12-hour light/dark cycle at the animal facilities of Massachusetts Institute of Technology, accredited by the American Association of Laboratory Animal Care. Both water and food were provided *ad libitum*.

2.4 Subcutaneous injection of polymeric microparticles

Before subcutaneous injection of microparticles, mice were kept under inhaled anesthesia using 1–4% isoflurane in oxygen at a flow rate of 2.5L/min. Lyophilized microparticles with or without encapsulated drug were suspended in sterile 0.9% (w/v) phosphate buffered saline at a concentration of 5mg/mL. A volume of 100µL of this suspension was injected subcutaneously via a 23G needle at each of the six spots on the back of the mouse.

2.5 In vivo fluorescent imaging of whole animal

Mice were started on a non-fluorescent alfalfa-free diet (Harlan Teklad, Madison, WI, USA) three days prior to subcutaneous injections of microparticles and maintained on this diet till the desired sacrifice time point for tissue harvesting. The imaging probe ProSense-680 (VisEn Medical, Woburn, MA, USA), at a concentration of 2nmol in 150µl of sterile phosphate buffered saline, was injected into the mice tail vein. After 24 hours, *in vivo* fluorescence imaging was performed with an IVIS-Spectrum measurement system (Xenogen, Hopkinton, MA, USA). The animals were maintained under inhaled anesthesia using 1–4% isoflurane in oxygen at a flow rate of 2.5L/min. For monitoring cathepsin activity, whole-animal near-infrared fluorescent images were captured at an excitation of 605nm and emission of 720nm and under optimized imaging configurations. A binning of 8×8 and a field of view of 13.1cm were used for imaging. Exposure time and f/stop (the opening size of the aperture) were optimized for each acquired image. Background autofluorescence of PLGA particles was also imaged at an excitation of 465nm and emission of 560nm. Data were analyzed using the manufacturer's Living Image 3.1 software. All images are presented in fluorescence efficiency which is defined as the ratio of the collected fluorescent intensity normalized against an internal reference to account for the variations in the distribution of incident light intensity. Regions of interest (ROIs) were determined around the site of injection. ROI signal intensities were calculated in fluorescent efficiency.

2.6 Tissue harvest and histology processing

At the desired time points, mice were euthanized via CO₂ asphyxiation. The injected microparticles and 1cm² area of full thickness dermal tissue surrounding the implant were excised, placed in histology cassettes and fixed in 10% formalin overnight. Following fixation, the tissues were dehydrated by transferring the cassettes to 70% ethanol solutions. The polymer particles with surrounding fixed tissues were embedded in paraffin and sectioned into samples of 5µm thickness. These samples were stained with hematoxylin and eosin (H&E) for histological analysis.

2.7 Histology analysis by laser scanning cytometry

The extent of cellular infiltration to injected polymer spots was determined by semi-quantitative imaging cytometry using the iCys Research Imaging Cytometer with iNovator software (CompuCyte, Cambridge, MA, USA). A scanning protocol for quantification was configured with excitation by blue 488nm laser and a virtual channel for hematoxylin detection. Low resolution tissue scans with the 20x objective were performed to capture preliminary images of all tissue sections in each slide. High resolution tissue scans were subsequently acquired using the 40x objective and step size of 0.5 μ m. The threshold in the hematoxylin channel for detection of cell nuclei was optimized to selectively contour individual nuclei. Cross-sectional areas of the polymer spots excluding the dermal and skeletal tissues were defined. The nuclei number and nuclei area measurements were taken from within these regions. The extent of cellular infiltration into each polymer spot was calculated as the ratio of the total nuclei area to total polymer cross-sectional area.

2.8 Statistical analysis

The values of the fluorescent signals and the extent of cellular infiltration were averaged and expressed as the mean $\pm$ standard error of the mean. Comparisons of values were performed by the Student's two-tailed two-sample *t*-test. *P* values less than 0.05 were considered significant.

3. Results and Discussion

3.1 Spatial effect of a controlled-release anti-inflammatory drug

3.1.1 Effect of drug loading on controlled-release properties—We first investigated the inhibitory effect of microparticles with different loadings of an anti-inflammatory drug. Dexamethasone, a synthetic steroid, was selected for incorporation into PLGA microparticles because it is the most potent long-acting glucocorticoid[40] which has been reported to decrease cellular recruitment to implanted biomaterials[17, 28, 31, 41] and to minimize fibrotic deposition on FDA-approved pace-maker leads[12]. PLGA particles with or without different drug loadings were fabricated by a water-in-oil emulsion method. Each formulation of drug-loaded particles was tested via subcutaneous injections at three alternating sites on the dorsal side of each mouse as shown in the injection scheme (Figure 1A). Control particles without encapsulated drug were similarly administered at the three remaining sites on the same mouse. Each mouse was imaged 24 hours after intravenous administration of Prosense680, a near-infrared fluorescent probe to detect the activity of cathepsin enzymes which are inflammatory proteases secreted by immune cells[42–44].

Figure 1B–C shows the imaging results at day 4 for two representative mice corresponding to two particle formulations with low (1.3wt%) and high (26wt%) drug loadings. For the mouse with low loading particles (Figure 1B), cathepsin activity of inflammatory cells was observed at three injection sites with control particles. This near-infrared fluorescent signal was absent for the drug-loaded particles at the remaining sites on the same mouse. The juxtaposition of cathepsin-absent sites next to cathepsin-active sites suggested that the anti-inflammatory effect was spatially localized at the injection sites of dexamethasone-loaded particles. Though the mechanism of action for dexamethasone is not completely understood,

it is known to act via a variety of pathways[33] resulting in the attenuation of inflammatory cell cascades when administered systemically[45]. *Ex vivo* histology studies also reported that this drug decreases fibroblastic recruitment and collagen production at implant sites[24]. Our data showed *in vivo* for the first time that controlled-release formulations of dexamethasone (1.3wt% drug loading) exhibited specific and localized inhibition of cathepsin activity in host response to subcutaneously implanted materials.

With the higher drug loading, there appeared to be a systemic immunosuppressant effect causing the disappearance of cathepsin signals from all six injection sites (Figure 1C). This might be due to the significant initial burst release from the particles with higher drug loading, as illustrated by the *in vitro* drug release profile (Figure 1D). Several mice administered with particles of high drug loading died after 7–10 days. Conversely, mice receiving particles with low drug loading maintained healthy body conditions till sacrifice at 28 days. Understanding the effect of drug loading on the *in vivo* inhibitory properties is important in selecting drug delivery formulations for incorporation into medical devices. Choosing an appropriate anti-inflammatory drug release profile may minimize unwanted side effects of systemic circulation, while ensuring sufficient mitigation of the host response to achieve long-term device performance.

3.1.2 Anti-inflammatory drug attenuated coverage of implanted polymer by immune cell layers—When we imaged the mice with low drug-loading particles at both near-infrared and green wavelength conditions (Figure 2), we discovered an interesting phenomenon relating to the optical properties of immune cell layers surrounding the injected polymer particles. Shown here are representative images from one mouse, displaying near-infrared (Figure 2B) and green (Figure 2C) fluorescent signals overlayed on a gray photograph at day 10 after subcutaneous injection of particles. Figure 2D shows the multiplex image combining the two fluorescent signals. Three injection sites with control particles showed fluorescence under near-infrared excitation, but no green signal when imaged at visible wavelengths. Conversely, for the remaining injection sites with drug-loaded particles, green fluorescence was detected but near-infrared signal was absent. This phenomenon was observed consistently in other mice. In addition, the fluorescent excitation-emission spectra of solid PLGA 50/50 particles *in vitro* (figure 2E) revealed that both control and drug-loaded microparticles gave strong autofluorescence in the green visible wavelengths but undetectable signals in the near-infrared region.

We hypothesized that the presence or absence of immune cell layers accounted for the difference in fluorescent signals observed in figure 2B and 2C. As illustrated in figure 2F, inflammatory cells were extensively recruited to the injection sites of control particles, covering them in compact cellular layers and reducing the green polymer auto-fluorescence by tissue scattering and absorption[46]. However, infiltration of immune cells to the drug-loaded particles was inhibited due to the anti-inflammatory effect of dexamethasone. The absence of immune cells enabled the detection of green autofluorescence from the polymer. No near-infrared autofluorescent signal was detected from the drug-loaded particles because PLGA 50/50 polymer does not autofluoresce in this region.

Our hypothesis was confirmed by *ex vivo* imaging of the excised polymer particles. Figures 2G–I show the excised mouse skin with the dermal-subcutaneous surface facing upwards, exposing the polymer microparticles to the imaging camera. A colored photograph of the same tissue (figure 2G) verified that the control polymer spots were compacted in extensive fibrotic tissue while the drug-loaded microparticles remained flattened against the skin, with minimal coverage by immune cell layers. In Figure 2H, the near-infrared cathepsin activity of the *ex vivo* tissue was consistent with the *in vivo* data. At the green wavelength condition in Figure 2I, *ex vivo* control particles also showed some auto-fluorescence that was not as intense as that of the drug-loaded particles. This weak auto-fluorescence of the control particles was not seen during *in vivo* imaging as the polymer spots were underneath the skin layer. Overall, our data suggest that inhibition of cathepsin activity by a controlled-release anti-inflammatory drug correlates with decreased coverage of the implanted particles by immune cell layers. Our observation regarding the optical properties of immune cell layers may be used to noninvasively monitor long term fibrosis in response to subcutaneously implanted materials.

3.2 Temporal effect of controlled-release anti-inflammatory drug

3.2.1 Time-evolution of cathepsin activity—The *in vivo* host response to implanted materials is a dynamic process that involves many different cell types and biological pathways. Neutrophils, monocytes and macrophages release cathepsins during the process of degranulation[47, 48]. To kinetically monitor the effect of controlled-release dexamethasone on the activity of these immune cells, cathepsin activity was imaged in mice administered with dexamethasone-loaded particles (1.3wt% drug loading) following the timeline in Figure 3A. The results for one representative animal at four different time points are shown in Figure 3B. Cathepsin activity in response to control PLGA 50/50 particles was highest at days 3 and 10, and decreased significantly at later time points. However, for the microparticles containing dexamethasone, such cellular activity was suppressed at earlier time points and remained absent over the entire period of 28 days. Quantification of the time-evolution of this cathepsin activity is presented in figure 3C showing statistically significant differences between the two particle formulations at days 3 and 10. This temporal analysis suggests that monitoring of cathepsin activity is useful in detecting the anti-inflammatory effect of controlled-release therapeutics in the early phase of host response.

3.2.2 Time-evolution of cellular infiltration—To understand how the temporal dynamics of *in vivo* cathepsin activity was related to time-dependent cellular infiltration between the implanted microparticles, we also performed standard histological analysis of excised tissues. Three mice were sacrificed at days 3, 10, 17 and 28 corresponding to the imaging time points in Figure 3. The excised polymer and surrounding tissues were fixed, processed histologically and stained with Hematoxylin and Eosin. Figure 4A shows representative tissue sections in which cell nuclei stained dark blue while collagen and cytoplasmic materials stained pink.

Qualitative evaluation of samples collected on days 3 and 10 revealed that the central portions of many polymer sections were detached during histology processing, while samples collected on days 17 and 27 remained intact. The non-homogenous properties of

dermal tissue containing polymer particles rendered it fragile during histological processing steps such as microtome sectioning and exposure to various organic solvents[49]. In the earlier phase of the foreign body response, cellular layers surrounding the implants might have been thinner and weaker; hence samples on days 3 and 10 were more prone to dissociation from the dermal tissue. In the later phase of days 17 and 27, wound healing might have already resolved[9] with the formation of strong fibrotic capsules containing the particles; and thus the samples became more resilient during histology processing.

Despite the lower quality of samples collected on days 3 and 10, we observed neutrophils infiltrating the spaces between polymer particles for both control and drug-loaded samples, and minimal collagen deposition. At the later time points of days 17 and 27, extensive macrophage infiltration and collagen deposition were observed throughout the polymer sections of control samples, while drug-loaded samples were free of cellular infiltration.

We used laser scanning cytometry to quantify the amount of inflammatory cells recruited to the polymer injection sites according to established protocols[25, 37, 50–52]. Figure 4B shows the extent of cellular infiltration into each polymer spot, calculated as the ratio of total nuclei area to total polymer cross-sectional area. The cellular coverage ratio was not statistically different for days 3 and 10, possibly due to the sample detachment at earlier time points. However, the extent of infiltration of inflammatory cells was significantly lower for drug-encapsulated polymers at later time points (days 17 and 27). Together, the histological data and fluorescent imaging provided complementary information to confirm that incorporation of dexamethasone decreased early protease activity and long-term cellular infiltration in the host response to subcutaneously implanted materials.

4. Conclusion

In this study, we have demonstrated the *in vivo* spatial and temporal host response to a subcutaneously-implanted, controlled-release anti-inflammatory drug formulation. Microparticles with low drug loading (1.3wt%) locally inhibited the activity of cathepsin enzymes from immune cells, while high drug loading formulation (26wt%) resulted in systemic immunosuppression. We also learned that incorporation of dexamethasone at a low loading (1.3wt%) attenuated the coverage of polymeric microparticles by immune cell layers. Temporal monitoring of the drug effect confirmed that incorporation of dexamethasone decreased early enzymatic activity and long-term cellular infiltration to implanted materials. Although only one drug was tested in our study, this strategy may potentially be extrapolated to investigate other existing drugs or to screen for new small molecules to expand the pool of anti-inflammatory drugs. Various parameters influencing drug release kinetics, such as particle size and polymer molecular weight, may also be explored. The ability to control the effects of anti-inflammatory therapeutics on the host response should aid in the design of microsphere systems for implanted biomedical devices including cardiovascular stents and glucose sensors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.

Effect of drug loading on the localization of anti-inflammatory properties. **(A)** Injection pattern showing administration sites of PLGA particles without (●) and with (■) dexamethasone. **(B)** Near-infrared fluorescent imaging showed a high level of cathepsins at the injection sites of control particles but localized inhibition of these enzymes at the sites of particles with low drug loading. **(C)** Inhibition of cathepsin activity at all injection sites was observed when particles with high drug loading were investigated. **(D)** *In vitro* release profiles of dexamethasone showed a more pronounced initial burst release from microparticles with high drug loading.

Fig 2.

Anti-inflammatory drug attenuated coverage of implanted polymer by immune cell layers (A) Injection pattern showing administration sites of PLGA particles without (●) and with (■) dexamethasone. (B–C) Fluorescent imaging of the same mouse at different wavelengths showed near-infrared signal of cathepsin activity (B) only at the sites of control particles and green polymer auto-fluorescence (C) only at the sites of drug-loaded particles (D) Multiplex image combining *in vivo* fluorescent signals at both wavelengths. (E) Fluorescent excitation-emission spectra of PLGA (50/50) microparticles in solid state showed significant green autofluorescence but no near-infrared autofluorescence. (F) Schematic illustration of the optical effect of immune cell layers. (G) Colored photograph of the excised skin tissue showed that control polymer particles were compacted in extensive fibrous tissue which reduced polymer auto-fluorescence at green wavelengths. The drug-loaded microparticles remained flattened against the skin with minimal cellular coverage thus retaining their auto-fluorescence. (H–I) Fluorescent imaging of *ex vivo* tissue at different wavelengths showed near-infrared cathepsin activity (H) consistent with *in vivo* data. At the green wavelength condition (I), *ex vivo* control particles also showed some auto-fluorescence of lower intensity than that of drug-loaded particles.

Fig 3.

Quantitative temporal monitoring of cathepsin activity. (A) Timeline of probe administration and imaging. (B) Near-infrared fluorescent images of one representative mouse over a period of 28 days demonstrated the inhibitory effect of dexamethasone at the earlier time points. All figures are of the same color scale. (C) Quantification of near-infrared fluorescent signals from four replicates showed that cathepsin activity at the sites of control microparticles was higher than drug-loaded microparticles at days 3 and 10. (**) indicates $P < 0.05$ by the Student's two-sample two-tailed t-test.

Fig 4.

Quantitative monitoring of cellular infiltration to the inter-particle spaces. (A) Representative histology sections of excised tissues containing PLGA particles with and without dexamethasone from different mice sacrificed at various time points. Scale bar represents 50um for all pictures. (B) Quantitative analysis of cellular infiltration by laser scanning cytometry showed the inhibitory effect of dexamethasone at later time points: days 17 and day 28. Extent of infiltration by inflammatory cells was defined as the ratio of total nuclei area to total polymer cross-sectional area. (**) indicates $P < 0.05$ by the Student's two-sample two-tailed t-test.