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Multiplexed Cytokine Detection of Interstitial Fluid Collected from Polymeric Hollow Tube Implants – A Feasibility Study

Xiangdan Wang¹, Michelle R. Lennartz², Daniel J. Loegering³, and Julie A. Stenken^{1*,†}

¹Department of Chemistry and Chemical Biology, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 Eighth Street, Troy, NY 12180-3590

²Center for Cell Biology and Cancer Research, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208-3479

³Center for Cardiovascular Sciences, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208-3479

Abstract

Cytokines are important cellular signaling proteins involved in inflammation, wound healing and are thought to direct the foreign body response to implanted materials. In this work, polyurethane tubes (25 mm length, 1.02 mm i.d., and 1.65 mm o.d.) were implanted into subcutaneous tissue of male Sprague-Dawley rats. The tubes served as the biomaterial and a means to collect the interstitial fluid that would be exchanged within the tube lumen and the surrounding tissue. After three and seven days, the tubes were explanted and cytokines in the fluid were quantified with a multiplexed cytokine immunoassay. Six cytokines, interleukin-1 β (IL-1 β), IL-4, IL-6, IL-10, macrophage chemoattractant protein-1 (MCP-1), and tumor necrosis factor- α (TNF- α), were simultaneously quantified. All cytokine concentrations with the exception of IL-4 and TNF- α ranged between low pg/mL to mid ng/mL levels. Neither TNF- α nor IL-4 was detected from any sample. These results illustrate the potential of using the tube materials combined with bead-based immunoassays as a direct method for *in vivo* collection of multiple cytokines in low microliter sample volumes for fixed day biomaterial implant studies.

Keywords

Cytokine; Polyurethane; Foreign body response

2.0 INTRODUCTION

All implanted biomaterials undergo a foreign body response initiated by the implantation process [1,2]. The foreign body response involves multiple stages including inflammation and fibrous capsule formation and is mediated by several chemical components including cytokines, eicosanoids, nitric oxide, and superoxide [3,4]. Cytokines, a family of signaling proteins (~ 6 to 80 kDa), are associated with many vital functions including directing the immune response in mammalian systems [5,6]. These proteins form the basis of a sophisticated

*Corresponding Author, Phone: +1 479-575-7018, Fax: +1 479-575-4049, Email: jstenken@uark.edu.

†Present address: Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR 72701.

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cellular communication network within the immune system [7]. Consequently, quantification of multiple cytokines instead of a single cytokine is important to biomaterials research since these proteins play a critical role in the initial inflammatory response that is believed to be involved with directing wound healing and ultimate outcome post implantation [8].

The interstitial fluid (ISF) provides a pathway for cellular contents to diffuse throughout a tissue space. The ISF also provides a means of chemical communication between cells and capillaries [9]. Direct cytokine protein analysis within the ISF is challenging for several reasons. The free volume of accessible fluid is limited because most of the interstitial fluid is trapped in the gel-like phase of the interstitial space [10]. This is due to the high concentrations of proteoglycans (e.g., heparan sulfate, chondroitin sulfate, etc.) that comprise the extracellular matrix causing the interstitial space to have a hydrogel-like consistency. Different sampling methods have been previously proposed for sampling ISF fluid and include micropipette [11], wick [12] and sponge techniques [13]. The limited microliter sample volume obtained restricts the application of these methods for multiplexed protein analysis. Coetzee, et al. used open-ended plastic tubes as an implantable capsule to collect ISF [10]. By implanting 2 cm-long-plastic tubes with an inner diameter of 4 mm and outer diameter of 6 mm, 250 μ L of ISF was sampled six weeks post implantation for total protein analysis. The study conducted by Coetzee *et al.* was more interested in total ISF protein content and not cytokine concentrations which would be orders of magnitude lower concentration. However, their approach to sampling interstitial fluid combined with modern chemical analysis methods for proteins provides a convenient method for fluid collection for cytokine analysis.

Since cytokines direct the cellular response to biomaterial implants, there has been significant biomedical interest in their quantification at the site of a biomaterial implant [1,2,14,15]. Many studies that have evaluated cytokine release in a response to different biomaterials have used monocyte-macrophage cell culture methods for several decades [3,16,17,18]. Although these studies provide valuable information, *in vivo* quantitation of cytokine release in the presence of different biomaterials is necessary to truly understand the immunological response to implanted biomaterials. Such protein measurements would provide essential chemical communication information to be used to identify and perhaps ultimately bioengineer materials that generate a foreign body response that would be desired on a case-by-case basis since many polymeric materials exist that are used in different applications.

Several types of *in vivo* methods have been described in the literature for evaluation of the foreign body response to implanted materials. In the widely used cage-implant approach [19, 20], cytokines have been sampled from stainless-steel cages containing a target biomaterial. Though control cages were used, the collected ISF might not be representative of the real conditions at the tissue/biomaterial interface since the biomaterials inside cages can not get direct contact with the surrounding tissue. Additionally, it is not possible to know where the sample was obtained relative to the cage or the material. More recently the use of air pouches within the peritoneal cavity [21] provides another way to evaluate responses to biomaterial in ISF. In this strategy, air pouches were lavaged with 1 mL of saline. It is difficult to know the real concentrations of cytokines in ISF since it was diluted to an unknown extent.

Microdialysis sampling has been used to collect cytokines from the skin under various conditions [22,23]. While these studies were not intended to test a type of material chemistry during implantation, they do illustrate that cytokines can be collected from an implantation site surrounding a polymeric membrane. The disadvantage with the microdialysis sampling approach is that cytokines are not recovered with 100% efficiency and thus calibration becomes a great concern.

An additional challenge with direct protein measurements within the ISF is the high volume of sample fluid (~100 μ L) typically required to perform an immunoassay for one cytokine. This limitation has been overcome by the numerous validated assays and platforms (e.g., Luminex and BD Pharmingen) that are available for performing highly sensitive and selective multiplexed cytokine analysis in volumes ranging from 5 to 50 μ L using bead-based immunoassays [24]. With these platforms, the theoretical number of analytes that can be quantified within a single low microliter sample is 100. However, most manufacturers offer the ability to measure no more than 25 cytokines in a single sample.

In this study, polyurethane tubes were implanted *in vivo* to serve both as the biomaterial and as a means to collect the ISF for cytokine analyses. The solid tubes have both ends open with no other holes or perforations. Implantation time for the polyurethane tubes was limited to 3 and 7 days. The choice of these days was based on knowing the acute inflammatory response lasts between minutes to days [1] and published reports of maximum macrophage infiltration occurring within 3 days [25]. This approach enables the sample collection as well as the ability to gain access to the cytokine production during the foreign body response to these materials. The volume within the tubes allowed for collection of greater than 10 μ L of fluid which was sufficient to perform duplicate multiplexed cytokine assays.

Cytokines believed to be important to the foreign body response have been classified as pro-inflammatory/pro-wound healing (IL-1 β), pro-inflammatory/anti-wound healing (IL-2, IL-6, IL-8 and TNF- α), anti-inflammatory/anti-wound healing (IL-10), and anti-inflammatory/pro-wound healing (IL-1 receptor agonist [IL-1ra], IL-4, IL-13, and transforming growth factor- β [TGF- β]) [19,26]. In addition to these classifications, other important cytokines to wound healing include MCP-1, macrophage inflammatory protein-1 α (MIP-1 α), and granulocyte/macrophage colony-stimulating factor (GM-CSF) [27,28,29]. Using this information as a guide, these studies focused on six cytokines IL-4, IL-6, IL-10, IL-1 β , MCP-1 and TNF- α , as proof of principle for this approach.

3.0 EXPERIMENTAL SECTION

3.1. Animals and Material Implantation

Male Sprague-Dawley rats (250–300 g, Taconic, NY) were used for all experiments. The rats have free access to water and food with a 12-hour on/ off light cycle prior to the experiments. The rats were anesthetized using isoflurane and kept warm using a rectal-temperature controlled heating pad (CMA Microdialysis, Inc. North Chelmsford, MA, USA) during the surgery. Polyurethane medical grade tubes (length 25 mm, inner diameter 1.02 mm and outer diameter 1.65 mm) from Instech Solomon (San Antonio, TX) were implanted in these experiments.

Before implantation, the tubes were sterilized by ethylene oxide gas for 1 hour and aerated for 12 hours to remove the residual ethylene oxide. The tubes were implanted two per animal on each side of the dorsal subcutis (Figure 1). A total of twelve rats were used in this study. Six rats were used for the three day implantation (twelve total tubes were implanted) and another six were used for the seven day implantation (twelve total tubes were implanted). Surgical procedures for each animal averaged 30 minutes. Three and seven days after implantation, the tubes were explanted, and the fluid samples inside the tubes were divided using 1 μ L of the sample for a total protein analysis with the remaining sample for cytokine analysis and then stored in a –80 °C freezer. Fluid could be removed from the tubes by a brisk shake. All animal procedures were approved by the Albany Medical College IACUC committee.

3.2. Protein Analysis

Total protein in the explanted fluid was determined using a Bradford Assay with bovine serum albumin (BSA) as the standard. Briefly, assays were performed in a 96-well microtiter plate (Falcon, Morristown, TN) containing 5 μ L samples of protease free BSA standards (Sigma-Aldrich, St. Louis, MO). The standard concentrations ranged from 25 μ g/mL to 200 μ g/mL to which 100 μ L of the Bradford reagent containing the Coomassie Brilliant Blue G-250 was added. Explanted fluid samples were diluted 200-fold with phosphate buffered saline (PBS), pH 7.4 that contained 145 mM NaCl, 7.75 mM Na₂HPO₄, and 2.67 mM NaH₂PO₄ to allow the protein concentration to be within the assay range. The plate was read using a Spectra Max™ 340 microplate reader (Molecular Devices Corporation, Sunnyvale, CA) at 595 nm.

3.3. Cytokine Analysis

Cytokine concentrations within the collected fluid were measured using a Luminex 100 Multiplex System (Luminex Corp, Austin, TX) with Rat Cytokine Lincoplex Kits (Linco Research, St. Charles, MO) containing bead-based immunoassays for interleukin-1 β (IL-1 β), interleukin-4 (IL-4), interleukin-6 (IL-6), macrophage chemoattractant protein-1 (MCP-1), tumor necrosis factor- α (TNF- α), and interleukin-10 (IL-10). All samples were analyzed using the manufacturer suggested protocols (www.millipore.com). Calibration curves were performed between 4.88 and 20,000 pg/mL. The limit of quantitation was determined to be 20 pg/mL. The limit of detection for this study was defined as the lowest standard concentration of 4.88 pg/mL above the blank (0 pg/mL). The standard curves were fitted using software provided for the Luminex instrument by Upstate Scientific (Lake Placid, NY).^{*} Each sample was measured in duplicate. All statistical analyses and the box and whiskers plot were performed using Origin 6.0 software.

4.0 RESULTS

Figure 2 shows a box and whiskers plot for the six cytokines measured from the fluid obtained from the explanted polyurethane tubes after three days (Figure 2a) and seven days (Figure 2b) implantation. In Figure 2a, concentrations of the two pro-inflammatory cytokines, IL-6 and IL-1 β range from 70 to 1490 pg/mL and 70 to 1200 pg/mL, respectively. Concentration of the chemokine, MCP-1 ranges from 1660 to 6250 pg/mL. Interestingly, IL-10, an anti-inflammatory cytokine was also present albeit at much lower concentrations (130 to 430 pg/mL). In contrast, the concentrations of the pro-inflammatory cytokine, TNF- α , and anti-inflammatory cytokine, IL-4 were below the limit of quantification (20 pg/mL) in all 12 samples.

The cytokine concentrations obtained for these tubes after seven days were similar to those as shown in Figure 2a. The concentration of IL-6 in ten out of twelve samples ranges from 220 to 6325 pg/mL with IL-6 in two samples below detection limit, 4.88 pg/mL. The concentration of IL-1 β ranged between 100 to 1185 pg/mL. For MCP-1, the concentration ranges from 1700 to 4960 pg/mL. IL-10 shows slightly lower concentrations (75 to 290 pg/mL) as compared to that after three days. Neither TNF- α nor IL-4 was detectable in all the 12 samples from the 7 day explants.

The total protein collected within the tube was measured as a means to correlate the results from the experiments. The mean protein concentrations for the tubes implanted three days vs. those implanted for seven days were 26.3 ± 2.4 (S.D.) mg/mL (n=12) and 29.9 ± 7.9 mg/mL (n=12). These concentrations are not statistically different (paired t-test) and are comparable

^{*}Upstate Scientific was recently acquired by Millipore.

to the total protein concentrations of approximately 30.2 mg/mL reported for rat interstitial fluid using the wick method [30].

5.0 DISCUSSION

5.1. Comparison of Cytokines Obtained from 3 vs. 7 Days

The cytokine concentrations recovered from the tubes implanted for three vs. seven days are not statistically different. However, larger variability in the concentrations obtained for IL-6 were observed. Tubes implanted for seven days appeared to be encapsulated as compared to the tubes explanted after three days. Although the three-day explanted tubes did not appear to be encapsulated, there appeared to be newly grown connective tissue embedded in the open ends of the tubes. It is likely that between three and seven days the ends of the tubes become occluded. With the exception of IL-10, there were no significant cytokine concentration differences between the implants for three vs. seven days at the 95% confidence level using a t-test. However, the lower concentration of the anti-inflammatory IL-10 with the seven-day data may suggest a slight change in the inflammatory state of the cells surrounding the biomaterial.

As mentioned in the introduction section, there have been few methods used to directly collected cytokines at their site of action. Microdialysis sampling has been used to collect IL-6 from the subcutaneous space from rats. In the microdialysis studies, the IL-6 concentrations had mean values of 470 pg/mL at 3-days and 290 pg/mL at 7-days post-implantation [23]. These mean values fall within the range of concentrations collected in this study with the open tubes.

5.2. Cytokine Discussion

A recent study analyzed the pattern of cytokine RNA in exudates collected from cage implants containing different biomaterials with a variety of surface chemistry, e.g., hydrophilic, hydrophobic, cationic and anionic. A polymerase chain reaction analysis revealed upregulation for IL-1 β , IL-4, IL-6, and IL-10 [19]. Additionally the seven-day exudates exhibited significantly higher cell mRNA expression of IL-1 β and IL-10 than IL-6 and IL-4. This strongly suggests that these four cytokines are associated with the foreign body response. The cytokine protein concentrations measured in this study exhibited a slightly different pattern of MCP-1 > IL-6 > IL-1 β > IL-10, with IL-4 and TNF- α concentrations below detection limits. It is worth noting that MCP-1 mRNA was not measured in the above-mentioned study. Since mRNA expression levels do not necessarily correlate with secreted protein [31], the multiplexed cytokine protein analysis is necessary to validate mRNA studies.

In this study, MCP-1 concentrations were the highest among all the cytokines which is in agreement with reports of high MCP-1 concentrations bound to inflammatory cells within biomaterials sites [32]. IL-1 β concentration in pouch fluid stimulated by different particles from air pouch membrane ranges from 200 to 480 pg/mL [21], which is similar to our observations. While this manuscript was under revision, a separate study using much larger polymeric materials (1 cm diameter by 1 cm in length) to collect cytokines from Wistar rats was published [33]. These authors reported similar IL-1 β , IL-6, and IL-10 concentrations to what we report at day 7.

In addition to undetectable concentrations of IL-4, the proinflammatory cytokine TNF- α was not detected in any of the explanted fluid. The inability to detect TNF- α is not due to the cytokine immunoassay kits as we have previously used these assays to quantify TNF- α *in vivo* and in macrophage cell culture [34,35]. The actual secreted TNF- α concentration may simply be too low to be detected noting that any detectable cytokine is actually an "overflow"

event since it has not bound to a cellular receptor. As in Wooley's study TNF- α concentrations stimulated by different particles in mice were all below 50 pg/ml [21]. The combinations of these reasons may explain the inability to detect TNF- α from the interstitial fluid explanted from the polymeric tubes.

6.0 SUMMARY

In conclusion, this preliminary study describes a direct sampling approach to identify and quantify expression of multiple cytokines from the interstitial fluid at the direct site of a biomaterial implant. The multiplexed cytokine measurements provide a crucial tool for studying the complex cytokine network interactions that drive the foreign body response. While the tubes provided a means to directly sample the interstitial fluid surrounding the biomaterial, the encapsulated tissue at the tube ends may affect cytokine diffusion and exchange with the surrounding tissue. This limits the application of this method to the early stage of foreign body response (~ 3 days). There are many additional design considerations for such studies as it is well known that different chemical compositions, level of surface roughness/morphology, and porosity affect the foreign body response and ultimately the biocompatibility of materials. Moreover, there many other signaling proteins that might be of interest to compare in an array format such as MIP-1 α and TGF- β . All of these potential studies are beyond the scope of this pilot study.

7.0 ACKNOWLEDGMENTS

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Figure 1.
Diagram for rat surgery. The parallel lines denote the direction of the implanted tubes into the dorsal subcutis.

Figure 2.

Cytokine profiles collected after three (2a) and seven days (2b) of implantation of the soft tubing from Instech Solomon. The filled squares (■) represent the mean of data, the box dimensions represent the 25–75th percentile of data, the bar in the middle is the 50th percentile of data, the error bars denote the 5–95th percentile of data, and the triangles (▲, ▼) represent the maximum and minimum values obtained in the data set. Data were collected from 12 tubes implanted into 6 rats (2 tubes per rat) for each set (three vs. seven days).